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(54) Title: 5' TO 3' EXONUCLEASE MUTATIONS OF THERMOSTABLE DNA POLYMERASES (57) Abstract The present invention relates to thermostable DNA polymerases which exhibit a different level of 5' to 3' exonuclease activity than their respective native polymerases. Particular conserved amino acid domains in thermostable DNA polymerases are mutated or deleted to alter the 5' to 3' exonuclease activity of the polymerases. The present invention also relates to means for isolating and producing such altered polymerases.		

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5' TO 3' EXONUCLEASE MUTATIONS OF
THERMOSTABLE DNA POLYMERASES

10

Cross-Reference to Related Applications

This is a continuation-in-part (CIP) of copending Serial Nos. 590,213, 590,466 and 590,490 all of which were filed on September 28, 1990, and all of which are CIPs of Serial No. 523,394, filed May 15, 1990, which is a CIP of abandoned Serial No. 143,441, filed January 12, 1988, which is a CIP of Serial No. 063,509, filed June 17, 1987, which issued as United States Patent No. 4,889,818 and which is a CIP of abandoned Serial No. 899,241, filed August 22, 1986.

This is also a CIP of Serial No. 746,121 filed August 15, 1991 which is a CIP of: 1) PCT/US90/07641, filed December 21, 1990, which is a CIP of Serial No. 585,471, filed September 20, 1990, which is a CIP of Serial No. 455,611, filed December 22, 1989, which is a CIP of Serial No. 143,441, filed January 12, 1988 and its ancestors as described above; and 2) Serial No. 609,157, filed November 2, 1990, which is a CIP of Serial No. 557,517, filed July 24, 1990.

This CIP is also related to the following patent applications:

U.S. Serial No. 523,394, filed May 15, 1990;
U.S. Serial No. 455,967, filed December 22, 1989;
PCT Application No. 91/05571, filed August 6, 1991;
PCT Application No. 91/05753, filed August 13, 1991.

All of the patent applications referenced in this section are incorporated herein by reference.

Background of the Invention

Field of the Invention

5 The present invention relates to thermostable DNA polymerases which have been altered or mutated such that a different level of 5' to 3' exonuclease activity is exhibited from that which is exhibited by the native enzyme. The present invention also relates to means
10 for isolating and producing such altered polymerases. Thermostable DNA polymerases are useful in many recombinant DNA techniques, especially nucleic acid amplification by the polymerase chain reaction (PCR) self-sustained sequence replication (3SR), and high
15 temperature DNA sequencing.

Background Art

Extensive research has been conducted on the
20 isolation of DNA polymerases from mesophilic microorganisms such as E. coli. See, for example, Bessman et al., 1957, J. Biol. Chem. 223:171-177 and Buttin and Kornberg, 1966, J. Biol. Chem. 241:5419-5427.

Somewhat less investigation has been made on the
25 isolation and purification of DNA polymerases from thermophiles such as Thermus aquaticus, Thermus thermophilus, Thermotoga maritima, Thermus species sps 17, Thermus species Z05 and Thermosipho africanus. The use of thermostable enzymes to amplify existing
30 nucleic acid sequences in amounts that are large compared to the amount initially present was described in United States Patent Nos. 4,683,195 and 4,683,202, which describe the PCR process, both disclosures of which are incorporated herein by reference. Primers,
35 template, nucleoside triphosphates, the appropriate buffer and reaction conditions, and polymerase are used

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in the PCR process, which involves denaturation of target DNA, hybridization of primers, and synthesis of complementary strands. The extension product of each primer becomes a template for the production of the
5 desired nucleic acid sequence. The two patents disclose that, if the polymerase employed is a thermostable enzyme, then polymerase need not be added after every denaturation step, because heat will not destroy the polymerase activity.

10 United States Patent No. 4,889,818, European Patent Publication No. 258,017 and PCT Publication No. 89/06691, the disclosures of which are incorporated herein by reference, all describe the isolation and recombinant expression of an ~94 kDa thermostable DNA
15 polymerase from Thermus aquaticus and the use of that polymerase in PCR. Although T. aquaticus DNA polymerase is especially preferred for use in PCR and other recombinant DNA techniques, there remains a need for other thermostable polymerases.

20

Summary of the Invention

In addressing the need for other thermostable polymerases, the present inventors found that some
25 thermostable DNA polymerases such as that isolated from Thermus aquaticus (Taq) display a 5' to 3' exonuclease or structure-dependent single-stranded endonuclease (SDSSE) activity. As is explained in greater detail below, such 5' to 3' exonuclease activity is un-
30 desirable in an enzyme to be used in PCR, because it may limit the amount of product produced and contribute to the plateau phenomenon in the normally exponential accumulation of product. Furthermore, the presence of
5' to 3' nuclease activity in a thermostable DNA polym-
35 erase may contribute to an impaired ability to efficiently generate long PCR products greater than or

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equal to 10 kb particularly for G+C-rich targets. In DNA sequencing applications and cycle sequencing applications, the presence of 5' to 3' nuclease activity may contribute to reduction in desired band intensities and/or generation of spurious or background bands. Finally, the absence of 5' to 3' nuclease activity may facilitate higher sensitivity allelic discrimination in a combined polymerase ligase chain reaction (PLCR) assay.

10 However, an enhanced or greater amount of 5' to 3' exonuclease activity in a thermostable DNA polymerase may be desirable in such an enzyme which is used in a homogeneous assay system for the concurrent amplification and detection of a target nucleic acid sequence. 15 Generally, an enhanced 5' to 3' exonuclease activity is defined as an enhanced rate of exonuclease cleavage or an enhanced rate of nick-translation synthesis or by the displacement of a larger nucleotide fragment before cleavage of the fragment.

20 Accordingly, the present invention was developed to meet the needs of the prior art by providing thermostable DNA polymerases which exhibit altered 5' to 3' exonuclease activity. Depending on the purpose for which the thermostable DNA polymerase will be used, the 25 5' to 3' exonuclease activity of the polymerase may be altered such that a range of 5' to 3' exonuclease activity may be expressed. This range of 5' to 3' exonuclease activity extends from an enhanced activity to a complete lack of activity. Although enhanced 30 activity is useful in certain PCR applications, e. g. a homogeneous assay, as little 5' to 3' exonuclease activity as possible is desired in thermostable DNA polymerases utilized in most other PCR applications.

 It was also found that both site directed 35 mutagenesis as well as deletion mutagenesis may result in the desired altered 5' to 3' exonuclease activity in

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the thermostable DNA polymerases of the present invention. Some mutations which alter the exonuclease activity have been shown to alter the processivity of the DNA polymerase. In many applications (e.g. amplification of moderate sized targets in the presence of a large amount of high complexity genomic DNA) reduced processivity may simplify the optimization of PCRs and contribute to enhanced specificity at high enzyme concentration. Some mutations which eliminate 5' to 3' exonuclease activity do not reduce and may enhance the processivity of the thermostable DNA polymerase and accordingly, these mutant enzymes may be preferred in other applications (e.g. generation of long PCR products). Some mutations which eliminate the 5' to 3' exonuclease activity simultaneously enhance, relative to the wild type, the thermoresistance of the mutant thermostable polymerase, and thus, these mutant enzymes find additional utility in the amplification of G+C-rich or otherwise difficult to denature targets.

Particular common regions or domains of thermostable DNA polymerase genomes have been identified as preferred sites for mutagenesis to affect the enzyme's 5' to 3' exonuclease. These domains can be isolated and inserted into a thermostable DNA polymerase having none or little natural 5' to 3' exonuclease activity to enhance its activity. Thus, methods of preparing chimeric thermostable DNA polymerases with altered 5' to 3' exonuclease are also encompassed by the present invention.

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Detailed Description of the Invention

The present invention provides DNA sequences and expression vectors that encode thermostable DNA polymerases which have been mutated to alter the

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expression of 5' to 3' exonuclease. To facilitate understanding of the invention, a number of terms are defined below.

5 The terms "cell", "cell line", and "cell culture" can be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary transformed cell and cultures derived from that cell without regard
10 to the number of transfers. All progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included in the definition of
15 transformants.

 The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for
20 procaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly other sequences. Eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

25 The term "expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect
30 transformation, the expression system may be included on a vector; however, the relevant DNA may also be integrated into the host chromosome.

 The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for
35 the production of a recoverable bioactive polypeptide or precursor. The polypeptide can be encoded by a full

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length coding sequence or by any portion of the coding sequence so long as the enzymatic activity is retained.

The term "operably linked" refers to the positioning of the coding sequence such that control sequences will function to drive expression of the protein encoded by the coding sequence. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequences can be expressed under the direction of a control sequence.

10 The term "mixture" as it relates to mixtures containing thermostable polymerases refers to a collection of materials which includes a desired thermostable polymerase but which can also include other proteins. If the desired thermostable polymerase 15 is derived from recombinant host cells, the other proteins will ordinarily be those associated with the host. Where the host is bacterial, the contaminating proteins will, of course, be bacterial proteins.

The term "non-ionic polymeric detergents" refers to 20 surface-active agents that have no ionic charge and that are characterized for purposes of this invention, by an ability to stabilize thermostable polymerase enzymes at a pH range of from about 3.5 to about 9.5, preferably from 4 to 8.5.

25 The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends 30 on the ultimate function or use of the oligonucleotide. The oligonucleotide may be derived synthetically or by cloning.

The term "primer" as used herein refers to an oligonucleotide which is capable of acting as a point 35 of initiation of synthesis when placed under conditions in which primer extension is initiated. An

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oligonucleotide "primer" may occur naturally, as in a purified restriction digest or be produced synthetically. Synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated in the presence of four different nucleoside triphosphates and a thermostable polymerase enzyme in an appropriate buffer at a suitable temperature. A "buffer" includes cofactors (such as divalent metal ions) and salt (to provide the appropriate ionic strength), adjusted to the desired pH.

A primer is single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer is usually an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerase enzyme. The exact length of a primer will depend on many factors, such as source of primer and result desired, and the reaction temperature must be adjusted depending on primer length and nucleotide sequence to ensure proper annealing of primer to template. Depending on the complexity of the target sequence, an oligonucleotide primer typically contains 15 to 35 nucleotides. Short primer molecules generally require lower temperatures to form sufficiently stable complexes with template.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially

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complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the 5 template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

The terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes which 10 cut double-stranded DNA at or near a specific nucleotide sequence.

The term "thermostable polymerase enzyme" refers to an enzyme which is stable to heat and is heat resistant and catalyzes (facilitates) combination of the 15 nucleotides in the proper manner to form primer extension products that are complementary to a template nucleic acid strand. Generally, synthesis of a primer extension product begins at the 3' end of the primer and proceeds in the 5' direction along the template 20 strand, until synthesis terminates.

In order to further facilitate understanding of the invention, specific thermostable DNA polymerase enzymes are referred to throughout the specification to exemplify the broad concepts of the invention, and 25 these references are not intended to limit the scope of the invention. The specific enzymes which are frequently referenced are set forth below with a common abbreviation which will be used in the specification and their respective nucleotide and amino acid Sequence 30 ID numbers.

<u>Thermostable DNA Polymerase</u>	<u>Common Abbr.</u>	<u>SEQ. ID NO:</u>
35 <u>Thermus aquaticus</u>	<u>Taq</u>	SEQ ID NO:1 (nuc)
		SEQ ID NO:2 (a.a.)

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	<u>Thermotoga maritima</u>	<u>Tma</u>	SEQ ID NO:3 (nuc)
			SEQ ID NO:4 (a.a.)
5	<u>Thermus species sps17</u>	<u>Tsps17</u>	SEQ ID NO:5 (nuc)
			SEQ ID NO:6 (a.a.)
10	<u>Thermus species Z05</u>	<u>TZ05</u>	SEQ ID NO:7 (nuc)
			SEQ ID NO:8 (a.a.)
15	<u>Thermus thermophilus</u>	<u>Tth</u>	SEQ ID NO:9 (nuc)
			SEQ ID NO:10 (a.a.)
	<u>Thermosipho africanus</u>	<u>Taf</u>	SEQ ID NO:11 (nuc)
20			SEQ ID NO:12 (a.a.)

As summarized above, the present invention relates to thermostable DNA polymerases which exhibit altered 5' to 3' exonuclease activity from that of the native polymerase. Thus, the polymerases of the invention exhibit either an enhanced 5' to 3' exonuclease activity or an attenuated 5' to 3' exonuclease activity from that of the native polymerase.

Thermostable DNA Polymerases With Attenuated 30 5' to 3' Exonuclease Activity

DNA polymerases often possess multiple functions. In addition to the polymerization of nucleotides E. coli DNA polymerase I (pol I), for example, catalyzes the pyrophosphorolysis of DNA as well as the hydrolysis of phosphodiester bonds. Two such hydrolytic activities have been characterized for pol I; one is a 3' to 5' exonuclease activity and the other a 5' to 3' exonuclease activity. The two exonuclease activities are associated with two different domains of the pol I molecule. However, the 5' to 3' exonuclease activity of pol I differs from that of thermostable DNA

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polymerases in that the 5' to 3' exonuclease activity of thermostable DNA polymerases has stricter structural requirements for the substrate on which it acts.

An appropriate and sensitive assay for the 5' to 3' exonuclease activity of thermostable DNA polymerases takes advantage of the discovery of the structural requirement of the activity. An important feature of the design of the assay is an upstream oligonucleoside primer which positions the polymerase appropriately for exonuclease cleavage of a labeled downstream oligonucleotide probe. For an assay of polymerization-independent exonuclease activity (i.e., an assay performed in the absence of deoxynucleoside triphosphates) the probe must be positioned such that the region of probe complementary to the template is immediately adjacent to the 3'-end of the primer. Additionally, the probe should contain at least one, but preferably 2-10, or most preferably 3-5 nucleotides at the 5'-end of the probe which are not complementary to the template. The combination of the primer and probe when annealed to the template creates a double stranded structure containing a nick with a 3'-hydroxyl 5' of the nick, and a displaced single strand 3' of the nick. Alternatively, the assay can be performed as a polymerization-dependent reaction, in which case each deoxynucleoside triphosphate should be included at a concentration of between 1 μ M and 2 mM, preferably between 10 μ M and 200 μ M, although limited dNTP addition (and thus limited dNTP inclusion) may be involved as dictated by the template sequence. When the assay is performed in the presence of dNTPs, the necessary structural requirements are an upstream oligonucleotide primer to direct the synthesis of the complementary strand of the template by the polymerase, and a labeled downstream oligonucleotide probe which will be contacted by the polymerase in the process of

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extending the upstream primer. An example of a polymerization-independent thermostable DNA polymerase 5' to 3' exonuclease assay follows.

The synthetic 3' phosphorylated oligonucleotide 5 probe (phosphorylated to preclude polymerase extension) BW33 (GATCGCTGCGCGTAACCAACACACCCGCGCGCp) (SEQ ID NO:13) (100 pmol) was ^{32}P -labeled at the 5' end with gamma- ^{32}P ATP (3000 Ci/mmol) and T4 polynucleotide kinase. The reaction mixture was extracted with 10 phenol:chloroform:isoamyl alcohol, followed by ethanol precipitation. The ^{32}P -labeled oligonucleotide probe was redissolved in 100 μl of TE buffer, and unincorporated ATP was removed by gel filtration chromatography on a Sephadex G-50 spin column. Five 15 pmol of ^{32}P -labeled BW33 probe, was annealed to 5 pmol of single-strand M13mp10w DNA, in the presence of 5 pmol of the synthetic oligonucleotide primer BW37 (GCGCTAGGGCGCTGGCAAGTGTAGCGGTCA) (SEQ ID NO:14) in a 100 μl reaction containing 10 mM Tris-HCl (pH 8.3), 20 50 mM KCl, and 3 mM MgCl_2 . The annealing mixture was heated to 95°C for 5 minutes, cooled to 70°C over 10 minutes, incubated at 70°C for an additional 10 minutes, and then cooled to 25°C over a 30 minute period in a Perkin-Elmer Cetus DNA Thermal Cycler. 25 Exonuclease reactions containing 10 μl of the annealing mixture were pre-incubated at 70°C for 1 minute. Thermostable DNA polymerase enzyme (approximately 0.01 to 1 unit of DNA polymerase activity, or 0.0005 to 0.05 pmol of enzyme) was added in a 2.5 μl volume to the 30 pre-incubation reaction, and the reaction mixture was incubated at 70°C. Aliquots (5 μl) were removed after 1 minute and 5 minutes, and stopped by the addition of 1 μl of 60 mM EDTA. The reaction products were analyzed by homochromatography and exonuclease activity 35 was quantified following autoradiography. Chromatography was carried out in a homochromatography

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mix containing 2% partially hydrolyzed yeast RNA in 7M urea on Polygram CEL 300 DEAE cellulose thin layer chromatography plates. The presence of 5' to 3' exonuclease activity results in the generation of small 5' ³²P-labeled oligomers, which migrate up the TLC plate, and are easily differentiated on the autoradiogram from undegraded probe, which remains at the origin.

The 5' to 3' exonuclease activity of the thermostable DNA polymerases excises 5' terminal 10 regions of double-stranded DNA releasing 5'-mono- and oligonucleotides in a sequential manner. The preferred substrate for the exonuclease is displaced single-stranded DNA, with hydrolysis of the phosphodiester bond occurring between the displaced single-stranded 15 DNA and the double-helical DNA. The preferred exonuclease cleavage site is a phosphodiester bond in the double helical region. Thus, the exonuclease activity can be better described as a structure-dependent single-stranded endonuclease 20 (SDSSE).

Many thermostable polymerases exhibit this 5' to 3' exonuclease activity, including the DNA polymerases of Taq, Tma, Tsps17, TZ05, Tth and Taf. When thermostable polymerases which have 5' to 3' exonuclease activity 25 are utilized in the PCR process, a variety of undesirable results have been observed including a limitation of the amount of product produced, an impaired ability to generate long PCR products or amplify regions containing significant secondary 30 structure, the production of shadow bands or the attenuation in signal strength of desired termination bands during DNA sequencing, the degradation of the 5'-end of oligonucleotide primers in the context of double-stranded primer-template complex, nick-

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translation synthesis during oligonucleotide-directed mutagenesis and the degradation of the RNA component of RNA:DNA hybrids.

The limitation of the amount of PCR product
5 produced is attributable to a plateau phenomenon in the otherwise exponential accumulation of product. Such a plateau phenomenon occurs in part because 5' to 3' exonuclease activity causes the hydrolysis or cleavage of phosphodiester bonds when a polymerase with 5' to 3'
10 exonuclease activity encounters a forked structure on a PCR substrate.

Such forked structures commonly exist in certain G- and C-rich DNA templates. The cleavage of these phosphodiester bonds under these circumstances is
15 undesirable as it precludes the amplification of certain G- and C-rich targets by the PCR process. Furthermore, the phosphodiester bond cleavage also contributes to the plateau phenomenon in the generation of the later cycles of PCR when product strand
20 concentration and renaturation kinetics result in forked structure substrates.

In the context of DNA sequencing, the 5' to 3' exonuclease activity of DNA polymerases is again a hinderance with forked structure templates because the
25 phosphodiester bond cleavage during the DNA extension reactions results in "false stops". These "false stops" in turn contribute to shadow bands, and in extreme circumstances may result in the absence of accurate and interpretable sequence data.

30 When utilized in a PCR process with double-stranded primer-template complex, the 5' to 3' exonuclease activity of a DNA polymerase may result in the degradation of the 5'-end of the oligonucleotide primers. This activity is not only undesirable in PCR,
35 but also in second-strand cDNA synthesis and sequencing processes.

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During optimally efficient oligonucleotide-directed mutagenesis processes, the DNA polymerase which is utilized must not have strand-displacement synthesis and/or nick-translation capability. Thus, the presence of 5' to 3' exonuclease activity in a polymerase used for oligonucleotide-directed mutagenesis is also undesirable.

Finally, the 5' to 3' exonuclease activity of polymerases generally also contains an inherent RNase H activity. However, when the polymerase is also to be used as a reverse transcriptase, as in a PCR process including an RNA:DNA hybrid, such an inherent RNase H activity may be disadvantageous.

Thus, one aspect of this invention involves the generation of thermostable DNA polymerase mutants displaying greatly reduced, attenuated or completely eliminated 5' to 3' exonuclease activity. Such mutant thermostable DNA polymerases will be more suitable and desirable for use in processes such as PCR, second-strand cDNA synthesis, sequencing and oligonucleotide-directed mutagenesis.

The production of thermostable DNA polymerase mutants with attenuated or eliminated 5' to 3' exonuclease activity may be accomplished by processes such as site-directed mutagenesis and deletion mutagenesis.

For example, a site-directed mutation of G to A in the second position of the codon for Gly at residue 46 in the Tag DNA polymerase amino acid sequence (i.e. mutation of G(137) to (A) in the DNA sequence has been found to result in an approximately 1000-fold reduction of 5' to 3' exonuclease activity with no apparent change in polymerase activity, processivity or extension rate. This site-directed mutation of the Tag DNA polymerase nucleotide sequence results in an amino acid change of Gly (46) to Asp.

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Glycine 46 of Tag DNA polymerase is conserved in Thermus species sps17 DNA polymerase, but is located at residue 43, and the same Gly to Asp mutation has a similar effect on the 5' to 3' exonuclease activity of 5 Tsps17 DNA polymerase. Such a mutation of the conserved Gly of Tth (Gly 46), TZ05 (Gly 46), Tma (Gly 37) and Taf (Gly 37) DNA polymerases to Asp also has a similar attenuating effect on the 5' to 3' exonuclease activities of those polymerases.

10 Tsps17 Gly 43, Tth Gly 46, TZ05 Gly 46, Tma Gly 37 and Taf Gly 37 are also found in a conserved A(V/T)YG (SEQ ID NO:15) sequence domain, and changing the glycine to aspartic acid within this conserved sequence domain of any polymerase is also expected to attenuate 15 5' to 3' exonuclease activity. Specifically, Tsps17 Gly 43, Tth Gly 46, TZ05 Gly 46, and Taf Gly 37 share the AVYG sequence domain, and Tma Gly 37 is found in the ATYG domain. Mutations of glycine to aspartic acid in other thermostable DNA polymerases containing the 20 conserved A(V/T)YG (SEQ ID NO:15) domain can be accomplished utilizing the same principles and techniques used for the site-directed mutagenesis of Tag polymerase. Exemplary of such site-directed mutagenesis techniques are Example 5 of U.S. Serial 25 No. 523,394, filed May 15, 1990, Example 4 of Attorney Docket No. 2583.1 filed September 27, 1991, Examples 4 and 5 of U.S. Serial No. 455,967, filed December 22, 1989 and Examples 5 and 8 of PCT Application No. 91/05753, filed August 13, 1991.

30 Such site-directed mutagenesis is generally accomplished by site-specific primer-directed mutagenesis. This technique is now standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to 35 be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the

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synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phasmid or phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. 5 Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage or plated on drug selective media for phasmid vectors.

Theoretically, 50% of the new plaques will contain 10 the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are transferred to nitrocellulose filters and the "lifts" hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact 15 match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then picked and cultured, and the DNA is recovered.

In the constructions set forth below, correct 20 ligations for plasmid construction are confirmed by first transforming E. coli strains DG98, DG101, DG116, or other suitable hosts, with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using 25 other markers, depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D.B., et al., Proc. Natl. Acad. Sci. (USA) (1969) 62:1159, optionally following 30 chloramphenicol amplification (Clewell, D.B., J. Bacteriol. (1972) 110: 667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., Proc. Natl. Acad. Sci. (USA) (1977) 74:5463 as further described by Messing,

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et al., Nucleic Acids Res. (1981) 9:309, or by the method of Maxam, et al., Methods in Enzymology (1980) 65:499.

For cloning and sequencing, and for expression of constructions under control of most lac or P_L promoters, E. coli strains DG98, DG101, DG116 were used as the host. For expression under control of the $P_{LN}RBS$ promoter, E. coli strain K12 MC1000 lambda lysogen, N₇N₅₃CI857 SusP₈₀, ATCC 39531 may be used.

10 Exemplary hosts used herein for expression of the thermostable DNA polymerases with altered 5' to 3' exonuclease activity are E. coli DG116, which was deposited with ATCC (ATCC 53606) on April 7, 1987 and E. coli KB2, which was deposited with ATCC (ATCC 53075)

15 on March 29, 1985.

For M13 phage recombinants, E. coli strains susceptible to phage infection, such as E. coli K12 strain DG98, are employed. The DG98 strain has been deposited with ATCC July 13, 1984 and has accession

20 number 39768.

Mammalian expression can be accomplished in COS-7 COS-A2, CV-1, and murine cells, and insect cell-based expression in Spodoptera frugiperda.

The thermostable DNA polymerases of the present invention are generally purified from E. coli strain DG116 containing the features of plasmid pLSG33. The primary features are a temperature regulated promoter (λ P_L promoter), a temperature regulated plasmid vector, a positive retro-regulatory element (PRE) (see

25 U.S. 4,666,848, issued May 19, 1987), and a modified form of a thermostable DNA polymerase gene. As described at page 46 of the specification of U.S patent application Serial No. 455,967, pLSG33 was prepared by ligating the NdeI-BamHI restriction fragment of pLSG24

30 into expression vector pDG178. The resulting plasmids are ampicillin resistant and capable of expressing 5'

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to 3' exonuclease deficient forms of the thermostable DNA polymerases of the present invention. The seed flask for a 10 liter fermentation contains tryptone (20 g/l), yeast extract (10 g/l), NaCl (10 g/l) and 0.005% 5 ampicillin. The seed flask is inoculated from colonies from an agar plate, or a frozen glycerol culture stock can be used. The seed is grown to between 0.5 and 1.0 O.D. (A_{680}). The volume of seed culture inoculated into the fermentation is calculated such that the final 10 concentration of bacteria will be 1 mg dry weight/liter. The 10 liter growth medium contained 25 mM KH_2PO_4 , 10 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM sodium citrate, 0.4 mM FeCl_2 , 0.04 mM ZnCl_2 , 0.03 mM CoCl_2 , 0.03 mM CuCl_2 , and 0.03 mM H_3BO_3 . The following sterile 15 components are added: 4 mM MgSO_4 , 20 g/l glucose, 20 mg/l thiamine-HCl and 50 mg/l ampicillin. The pH was adjusted to 6.8 with NaOH and controlled during the fermentation by added NH_4OH . Glucose is continually added during the fermentation by coupling to NH_4OH 20 addition. Foaming is controlled by the addition of polypropylene glycol as necessary, as an anti-foaming agent. Dissolved oxygen concentration is maintained at 40%.

The fermentation is inoculated as described above 25 and the culture is grown at 30°C until an optical density of 21 (A_{680}) is reached. The temperature is then raised to 37°C to induce synthesis of the desired polymerase. Growth continues for eight hours after induction, and the cells are then harvested by 30 concentration using cross flow filtration followed by centrifugation. The resulting cell paste is frozen at -70°C and yields about 500 grams of cell paste. Unless otherwise indicated, all purification steps are conducted at 4°C.

35 A portion of the frozen (-70°C) *E. coli* K12 strain DG116 harboring plasmid pLSG33 or other suitable host

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as described above is warmed overnight to -20°C . To the cell pellet the following reagents are added: 1 volume of 2X TE (100 mM Tris-HCl, pH 7.5, 20 mM EDTA), 1 mg/ml leupeptin and 144 mM PMSF (in dimethyl 5 formamide). The final concentration of leupeptin was 1 $\mu\text{g}/\text{ml}$ and for PMSF, 2.4 mM. Preferably, dithiothreitol (DTT) is included in TE to provide a final concentration of 1 mM DTT. The mixture is homogenized at low speed in a blender. All glassware 10 is baked prior to use, and solutions used in the purification are autoclaved, if possible, prior to use. The cells are lysed by passage twice through a Microfluidizer at 10,000 psi.

The lysate is diluted with 1X TE containing 1 mM 15 DTT to a final volume of 5.5X cell wet weight. Leupeptin is added to 1 $\mu\text{g}/\text{ml}$ and PMSF is added to 2.4 mM. The final volume (Fraction I) is approximately 1540 ml.

Ammonium sulfate is gradually added to 0.2 M (26.4 20 g/l) and the lysate stirred. Upon addition of ammonium sulfate, a precipitate forms which is removed prior to the polyethylenimine (PEI) precipitation step, described below. The ammonium sulfate precipitate is removed by centrifugation of the suspension at 15,000 - 25 20,000 xg in a JA-14 rotor for 20 minutes. The supernatant is decanted and retained. The ammonium sulfate supernatant is then stirred on a heating plate until the supernatant reaches 75°C and then is placed in a 77°C bath and held there for 15 minutes with 30 occasional stirring. The supernatant is then cooled in an ice bath to 20°C and a 10 ml aliquot is removed for PEI titration.

PEI titration and agarose gel electrophoresis are used to determine that 0.3% PEI (commercially available 35 from BDH as PolymixP) precipitates ~90% of the macromolecular DNA and RNA, i.e., no DNA band is

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visible on an ethidium bromide stained agarose gel after treatment with PEI. PEI is added slowly with stirring to 0.3% from a 10% stock solution. The PEI treated supernatant is centrifuged at 10,000 RPM 5 (17,000 xg) for 20 minutes in a JA-14 rotor. The supernatant is decanted and retained. The volume (Fraction II) is approximately 1340 ml.

Fraction II is loaded onto a 2.6 x 13.3 cm (71 ml) phenyl sepharose CL-4B (Pharmacia-LKB) column following 10 equilibration with 6 to 10 column volumes of TE containing 0.2 M ammonium sulfate. Fraction II is then loaded at a linear flow rate of 10 cm/hr. The flow rate is 0.9 ml/min. The column is washed with 3 column volumes of the equilibration buffer and then with 2 15 column volumes of TE to remove contaminating non-DNA polymerase proteins. The recombinant thermostable DNA polymerase is eluted with 4 column volumes of 2.5 M urea in TE containing 20% ethylene glycol. The DNA polymerase containing fractions are identified by 20 optical absorption (A_{280}), DNA polymerase activity assay and SDS-PAGE according to standard procedures. Peak fractions are pooled and filtered through a 0.2 micron sterile vacuum filtration apparatus. The volume (Fraction III) is approximately 195 ml. The resin is 25 equilibrated and recycled according to the manufacturer's recommendations.

A 2.6 x 1.75 cm (93 ml) heparin sepharose Cl-6B column (Pharmacia-LKB) is equilibrated with 6-10 column volumes of 0.05 M KCl, 50 mM Tris-HCl, pH 7.5, 0.1 mM 30 EDTA and 0.2% Tween 20, at 1 column volume/hour. Preferably, the buffer contains 1 mM DTT. The column is washed with 3 column volumes of the equilibration buffer. The desired thermostable DNA polymerase of the invention is eluted with a 10 column volume linear 35 gradient of 50-750 mM KCl gradient in the same buffer. Fractions (one-tenth column volume) are collected in

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sterile tubes and the fractions containing the desired thermostable DNA polymerase are pooled (Fraction IV, volume 177 ml).

Fraction IV is concentrated to 10 ml on an Amicon 5 YM30 membrane. For buffer exchange, diafiltration is done 5 times with 2.5X storage buffer (50 mM Tris-HCl, pH 7.5, 250 mM KCl, 0.25 mM EDTA 2.5 mM DTT and 0.5% Tween-20) by filling the concentrator to 20 ml and concentrating the volumes to 10 ml each time. The 10 concentrator is emptied and rinsed with 10 ml 2.5X storage buffer which is combined with the concentrate to provide Fraction V.

Anion exchange chromatography is used to remove residual DNA. The procedure is conducted in a 15 biological safety hood and sterile techniques are used. A Waters Sep-Pak plus QMA cartridge with a 0.2 micron sterile disposable syringe tip filter unit is equilibrated with 30 ml of 2.5X storage buffer using a syringe at a rate of about 5 drops per second. Using a 20 disposable syringe, Fraction V is passed through the cartridge at about 1 drop/second and collected in a sterile tube. The cartridge is flushed with 5 ml of 2.5 ml storage buffer and pushed dry with air. The eluant is diluted 1.5 X with 80% glycerol and stored at 25 -20°C. The resulting final Fraction IV pool contains active thermostable DNA polymerase with altered 5' to 3' exonuclease activity.

In addition to site-directed mutagenesis of a nucleotide sequence, deletion mutagenesis techniques 30 may also be used to attenuate the 5' to 3' exonuclease activity of a thermostable DNA polymerase. One example of such a deletion mutation is the deletion of all amino terminal amino acids up to and including the glycine in the conserved A(V/T)YG (SEQ ID NO:15) domain 35 of thermostable DNA polymerases.

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A second deletion mutation affecting 5' to 3' exonuclease activity is a deletion up to Ala 77 in Tag DNA polymerase. This amino acid (Ala 77) has been identified as the amino terminal amino acid in an approximately 85.5 kDa proteolytic product of Tag DNA polymerase. This proteolytic product has been identified in several native Tag DNA polymerase preparations and the protein appears to be stable. Since such a deletion up to Ala 77 includes Gly 46, it will also affect the 5' to 3' exonuclease activity of Tag DNA polymerase.

However, a deletion mutant beginning with Ala 77 has the added advantage over a deletion mutant beginning with phenylalanine 47 in that the proteolytic evidence suggests that the peptide will remain stable. Furthermore, Ala 77 is found within the sequence HEAYG (SEQ ID NO:16) 5 amino acids prior to the sequence YKA in Tag DNA polymerase. A similar sequence motif HEAYE (SEQ ID NO:17) is found in Tth DNA polymerase, TZ05 DNA polymerase and Tsps17 DNA polymerase. The alanine is 5 amino acids prior to the conserved motif YKA. The amino acids in the other exemplary thermostable DNA polymerases which correspond to Tag Ala 77 are Tth Ala 78, TZ05 Ala 78, Tsps17 Ala 74, Tma Leu 72 and Taf Ile 73. A deletion up to the alanine or corresponding amino acid in the motif HEAY(G/E) (SEQ ID NO:16 or SEQ ID NO:17) in a Thermus species thermostable DNA polymerase containing this sequence will attenuate its 5' to 3' exonuclease activity. The 5' to 3' exonuclease motif YKA is also conserved in Tma DNA polymerase (amino acids 76-78) and Taf DNA polymerase (amino acids 77-79). In this thermostable polymerase family, the conserved motif (L/I)LET (SEQ ID NO:18) immediately proceeds the YKA motif. Taf DNA polymerase Ile 73 is 5 residues prior to this YKA motif while TMA DNA polymerase Leu 72 is 5 residues prior to the YKA

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motif. A deletion of the Leu or Ile in the motif (L/I)LETYKA (SEQ ID NO:19) in a thermostable DNA polymerase from the Thermotoga or Thermosipho genus will also attenuate 5' to 3' exonuclease activity.

5 Thus, a conserved amino acid sequence which defines the 5' to 3' exonuclease activity of DNA polymerases of the Thermus genus as well as those of Thermotoga and Thermosipho has been identified as (I/L/A)X₃YKA (SEQ ID NO:20), wherein X₃ is any sequence of three amino
10 acids. Therefore, the 5' to 3' exonuclease activity of thermostable DNA polymerases may also be altered by mutating this conserved amino acid domain.

Those of skill in the art recognize that when such a deletion mutant is to be expressed in recombinant
15 host cells, a methionine codon is usually placed at the 5' end of the coding sequence, so that the amino terminal sequence of the deletion mutant protein would be MET-ALA in the Thermus genus examples above.

The preferred techniques for performing deletion
20 mutations involve utilization of known restriction sites on the nucleotide sequence of the thermostable DNA polymerase. Following identification of the particular amino acid or amino acids which are to be deleted, a restriction site is identified which when
25 cleaved will cause the cleavage of the target DNA sequence at a position or slightly 3' distal to the position corresponding to the amino acid or domain to be deleted, but retains domains which code for other properties of the polymerase which are desired.

30 Alternatively, restriction sites on either side (5' or 3') of the sequence coding for the target amino acid or domain may be utilized to cleave the sequence. However, a ligation of the two desired portions of the sequence will then be necessary. This ligation may be
35 performed using techniques which are standard in the art and exemplified in Example 9 of Serial No. 523,394,

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filed May 15, 1990, Example 7 of PCT Application No. 91/05753, filed August 13, 1991 and Serial No. 590,490, filed September 28, 1990, all of which are incorporated herein by reference.

5 Another technique for achieving a deletion mutation of the thermostable DNA polymerase is by utilizing the PCR mutagenesis process. In this process, primers are prepared which incorporate a restriction site domain and optionally a methionine codon if such a codon is
10 not already present. Thus, the product of the PCR with this primer may be digested with an appropriate restriction enzyme to remove the domain which codes for 5' to 3' exonuclease activity of the enzyme. Then, the two remaining sections of the product are ligated to
15 form the coding sequence for a thermostable DNA polymerase lacking 5' to 3' exonuclease activity. Such coding sequences can be utilized as expression vectors in appropriate host cells to produce the desired thermostable DNA polymerase lacking 5' to 3'
20 exonuclease activity.

In addition to the Taq DNA polymerase mutants with reduced 5' to 3' exonuclease activity, it has also been found that a truncated Tma DNA polymerase with reduced 5' to 3' exonuclease activity may be produced by
25 recombinant techniques even when the complete coding sequence of the Tma DNA polymerase gene is present in an expression vector in E. coli. Such a truncated Tma DNA polymerase is formed by translation starting with the methionine codon at position 140. Furthermore,
30 recombinant means may be used to produce a truncated polymerase corresponding to the protein produced by initiating translation at the methionine codon at position 284 of the Tma coding sequence.

The Tma DNA polymerase lacking amino acids 1 through
35 139 (about 86 kDa), and the Tma DNA polymerase lacking amino acids 1 through 283 (about 70 kDa) retain

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polymerase activity but have attenuated 5' to 3' exonuclease activity. An additional advantage of the 70 kDa Tma DNA polymerase is that it is significantly more thermostable than native Tma polymerase.

5 Thus, it has been found that the entire sequence of the intact Tma DNA polymerase I enzyme is not required for activity. Portions of the Tma DNA polymerase I coding sequence can be used in recombinant DNA techniques to produce a biologically active gene
10 product with DNA polymerase activity.

Furthermore, the availability of DNA encoding the Tma DNA polymerase sequence provides the opportunity to modify the coding sequence so as to generate mutein (mutant protein) forms also having DNA polymerase
15 activity but with attenuated 5' to 3' exonuclease activity. The amino(N)-terminal portion of the Tma DNA polymerase is not necessary for polymerase activity but rather encodes the 5' to 3' exonuclease activity of the protein.

20 Thus, using recombinant DNA methodology, one can delete approximately up to one-third of the N-terminal coding sequence of the Tma gene, clone, and express a gene product that is quite active in polymerase assays but, depending on the extent of the deletion, has no 5'
25 to 3' exonuclease activity. Because certain N-terminal shortened forms of the polymerase are active, the gene constructs used for expression of these polymerases can include the corresponding shortened forms of the coding sequence.

30 In addition to the N-terminal deletions, individual amino acid residues in the peptide chain of Tma DNA polymerase or other thermostable DNA polymerases may be modified by oxidation, reduction, or other derivation, and the protein may be cleaved to obtain fragments that
35 retain polymerase activity but have attenuated 5' to 3' exonuclease activity. Modifications to the primary

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structure of the Tma DNA polymerase coding sequence or the coding sequences of other thermostable DNA polymerases by deletion, addition, or alteration so as to change the amino acids incorporated into the 5 thermostable DNA polymerase during translation of the mRNA produced from that coding sequence can be made without destroying the high temperature DNA polymerase activity of the protein.

Another technique for preparing thermostable DNA 10 polymerases containing novel properties such as reduced or enhanced 5' to 3' exonuclease activity is a "domain shuffling" technique for the construction of "thermostable chimeric DNA polymerases". For example, substitution of the Tma DNA polymerase coding sequence 15 comprising codons about 291 through about 484 for the Tag DNA polymerase I codons 289-422 would yield a novel thermostable DNA polymerase containing the 5' to 3' exonuclease domain of Tag DNA polymerase (1-289), the 3' to 5' exonuclease domain of Tma DNA polymerase 20 (291-484), and the DNA polymerase domain of Tag DNA polymerase (423-832). Alternatively, the 5' to 3' exonuclease domain and the 3' to 5' exonuclease domains of Tma DNA polymerase (ca. codons 1-484) may be fused to the DNA polymerase (dNTP binding and primer/template 25 binding domains) portions of Tag DNA polymerase (ca. codons 423-832).

As is apparent, the donors and recipients for the creation of "thermostable chimeric DNA polymerase" by "domain shuffling" need not be limited to Tag and Tma 30 DNA polymerases. Other thermostable polymerases provide analogous domains as Tag and Tma DNA polymerases. Furthermore, the 5' to 3' exonuclease domain may derive from a thermostable DNA polymerase with altered 5' to 3' nuclease activity. For example, 35 the 1 to 289 5' to 3' nuclease domain of Tag DNA polymerase may derive from a Gly (46) to Asp mutant

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form of the Tag polymerase gene. Similarly, the 5' to 3' nuclease and 3' to 5' nuclease domains of Tma DNA polymerase may encode a 5' to 3' exonuclease deficient domain, and be retrieved as a Tma Gly (37) to Asp amino acid 1 to 484 encoding DNA fragment or alternatively a truncated Met 140 to amino acid 484 encoding DNA fragment.

While any of a variety of means may be used to generate chimeric DNA polymerase coding sequences (possessing novel properties), a preferred method employs "overlap" PCR. In this method, the intended junction sequence is designed into the PCR primers (at their 5'-ends). Following the initial amplification of the individual domains, the various products are diluted (ca. 100 to 1000-fold) and combined, denatured, annealed, extended, and then the final forward and reverse primers are added for an otherwise standard PCR.

Those of skill in the art recognize that the above thermostable DNA polymerases with attenuated 5' to 3' exonuclease activity are most easily constructed by recombinant DNA techniques. When one desires to produce one of the mutant enzymes of the present invention, with attenuated 5' to 3' exonuclease activity or a derivative or homologue of those enzymes, the production of a recombinant form of the enzyme typically involves the construction of an expression vector, the transformation of a host cell with the vector, and culture of the transformed host cell under conditions such that expression will occur.

To construct the expression vector, a DNA is obtained that encodes the mature (used here to include all chimeras or muteins) enzyme or a fusion of the mutant polymerase to an additional sequence that does not destroy activity or to an additional sequence cleavable under controlled conditions (such as treatment with peptidase) to give an active protein.

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The coding sequence is then placed in operable linkage with suitable control sequences in an expression vector. The vector can be designed to replicate autonomously in the host cell or to integrate into the 5 chromosomal DNA of the host cell. The vector is used to transform a suitable host, and the transformed host is cultured under conditions suitable for expression of the recombinant polymerase.

Each of the foregoing steps can be done in a 10 variety of ways. For example, the desired coding sequence may be obtained from genomic fragments and used directly in appropriate hosts. The construction for expression vectors operable in a variety of hosts is made using appropriate replicons and control 15 sequences, as set forth generally below. Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques that are well understood in the art. Isolated plasmids, DNA sequences, or synthesized 20 oligonucleotides are cleaved, modified, and religated in the form desired. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to facilitate construction of an expression vector, as exemplified below.

25 Site-specific DNA cleavage is performed by treating with suitable restriction enzyme (or enzymes) under conditions that are generally understood in the art and specified by the manufacturers of commercially available restriction enzymes. See, e.g., New England 30 Biolabs, Product Catalog. In general, about 1 μ g of plasmid or other DNA is cleaved by one unit of enzyme in about 20 μ l of buffer solution; in the examples below, an excess of restriction enzyme is generally used to ensure complete digestion of the DNA. 35 Incubation times of about one to two hours at about 37°C are typical, although variations can be

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tolerated. After each incubation, protein is removed by extraction with phenol and chloroform; this extraction can be followed by ether extraction and recovery of the DNA from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. See, e.g., Methods in Enzymology, 1980, 65:499-560.

10 Restriction-cleaved fragments with single-strand "overhanging" termini can be made blunt-ended (double-strand ends) by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleoside triphosphates
15 (dNTPs) using incubation times of about 15 to 25 minutes at 20°C to 25°C in 50 mM Tris-Cl pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 10 mM DTT, and 5 to 10 μM dNTPs. The Klenow fragment fills in at 5' protruding ends, but chews back protruding 3' single strands, even though
20 the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the protruding ends. After treatment with Klenow, the mixture is extracted with
25 phenol/chloroform and ethanol precipitated. Similar results can be achieved using S1 nuclease, because treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion of a nucleic acid.

30 Synthetic oligonucleotides can be prepared using the triester method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185-3191, or automated synthesis methods. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g.,
35 approximately 10 units, of polynucleotide kinase to 0.5 μM substrate in the presence of 50 mM Tris, pH 7.6,

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10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1 to 2 μM ATP. If kinasing is for labeling of probe, the ATP will contain high specific activity γ - ^{32}P .

Ligations are performed in 15-30 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 $\mu g/ml$ BSA, 10 mM-50 mM NaCl, and either 40 μM ATP and 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for ligation of fragments with complementary single-stranded ends) or 1 mM ATP and 0.3-0.6 units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular ligations of fragments with complementary ends are usually performed at 33-100 $\mu g/ml$ total DNA concentrations (5 to 100 nM total ends concentration). Intermolecular blunt end ligations (usually employing a 20 to 30 fold molar excess of linkers, optionally) are performed at 1 μM total ends concentration.

In vector construction, the vector fragment is commonly treated with bacterial or calf intestinal alkaline phosphatase (BAP or CIAP) to remove the 5' phosphate and prevent religation and reconstruction of the vector. BAP and CIAP digestion conditions are well known in the art, and published protocols usually accompany the commercially available BAP and CIAP enzymes. To recover the nucleic acid fragments, the preparation is extracted with phenol-chloroform and ethanol precipitated to remove the phosphatase and purify the DNA. Alternatively, religation of unwanted vector fragments can be prevented by restriction enzyme digestion before or after ligation, if appropriate restriction sites are available.

For portions of vectors or coding sequences that require sequence modifications, a variety of site-specific primer-directed mutagenesis methods are available. The polymerase chain reaction (PCR) can be

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used to perform site-specific mutagenesis. In another technique now standard in the art, a synthetic oligonucleotide encoding the desired mutation is used as a primer to direct synthesis of a complementary
5 nucleic acid sequence of a single-stranded vector, such as pBS13+, that serves as a template for construction of the extension product of the mutagenizing primer. The mutagenized DNA is transformed into a host bacterium, and cultures of the transformed bacteria are
10 plated and identified. The identification of modified vectors may involve transfer of the DNA of selected transformants to a nitrocellulose filter or other membrane and the "lifts" hybridized with kinased synthetic primer at a temperature that permits
15 hybridization of an exact match to the modified sequence but prevents hybridization with the original strand. Transformants that contain DNA that hybridizes with the probe are then cultured and serve as a reservoir of the modified DNA.

20 In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain DG101 or another suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline
25 or other antibiotic resistance or sensitivity or by using other markers, depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell et al., 1969, Proc. Natl. Acad.
30 Sci. USA 62:1159, optionally following chloramphenicol amplification (Clewell, 1972, J. Bacteriol. 110:667). Another method for obtaining plasmid DNA is described as the "Base-Acid" extraction method at page 11 of the Bethesda Research Laboratories publication Focus,
35 volume 5, number 2, and very pure plasmid DNA can be obtained by replacing steps 12 through 17 of the

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protocol with CsCl/ethidium bromide ultracentrifugation of the DNA. The isolated DNA is analyzed by restriction enzyme digestion and/or sequenced by the dideoxy method of Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74:5463, as further described by Messing et al., 1981, Nuc. Acids Res. 9:309, or by the method of Maxam et al., 1980, Methods in Enzymology 65:499.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Generally, procaryotic, yeast, insect, or mammalian cells are used as hosts. Procaryotic hosts are in general the most efficient and convenient for the production of recombinant proteins and are therefore preferred for the expression of the thermostable DNA polymerases of the present invention.

The procaryote most frequently used to express recombinant proteins is E. coli. For cloning and sequencing, and for expression of constructions under control of most bacterial promoters, E. coli K12 strain MM294, obtained from the E. coli Genetic Stock Center under GCSC #6135, can be used as the host. For expression vectors with the P_{LN}RBS control sequence, E. coli K12 strain MC1000 lambda lysogen, N₇N₅₃CI₈₅₇ SusP₈₀, ATCC 39531, may be used. E. coli DG116, which was deposited with the ATCC (ATCC 53606) on April 7, 1987, and E. coli KB2, which was deposited with the ATCC (ATCC 53075) on March 29, 1985, are also useful host cells. For M13 phage recombinants, E. coli strains susceptible to phage infection, such as E. coli K12 strain DG98, are employed. The DG98 strain was deposited with the ATCC (ATCC 39768) on July 13, 1984.

However, microbial strains other than E. coli can also be used, such as bacilli, for example Bacillus subtilis, various species of Pseudomonas, and other bacterial strains, for recombinant expression of the

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thermostable DNA polymerases of the present invention. In such procaryotic systems, plasmid vectors that contain replication sites and control sequences derived from the host or a species compatible with the host are typically used.

For example, E. coli is typically transformed using derivatives of pBR322, described by Bolivar et al., 1977, Gene 2:95. Plasmid pBR322 contains genes for ampicillin and tetracycline resistance. These drug resistance markers can be either retained or destroyed in constructing the desired vector and so help to detect the presence of a desired recombinant. Commonly used procaryotic control sequences, i.e., a promoter for transcription initiation, optionally with an operator, along with a ribosome binding site sequence, include the β -lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., 1977, Nature 198:1056), the tryptophan (trp) promoter system (Goeddel et al., 1980, Nuc. Acids Res. 8:4057), and the lambda-derived P_L promoter (Shimatake et al., 1981, Nature 292:128) and N-gene ribosome binding site (N_{RBS}). A portable control system cassette is set forth in United States Patent No. 4,711,845, issued December 8, 1987. This cassette comprises a P_L promoter operably linked to the N_{RBS} in turn positioned upstream of a third DNA sequence having at least one restriction site that permits cleavage within six bp 3' of the N_{RBS} sequence. Also useful is the phosphatase A (phoA) system described by Chang et al. in European Patent Publication No. 196,864, published October 8, 1986. However, any available promoter system compatible with procaryotes can be used to construct a modified thermostable DNA polymerase expression vector of the invention.

In addition to bacteria, eucaryotic microbes, such as yeast, can also be used as recombinant host cells.

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Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most often used, although a number of other strains are commonly available. While vectors employing the two micron origin of replication are common (Broach, 1983, Meth. Enz. 101:307), other plasmid vectors suitable for yeast expression are known (see, for example, Stinchcomb et al., 1979, Nature 282:39; Tschempe et al., 1980, Gene 10:157; and Clarke et al., 1983, Meth. Enz. 101:300). Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., 1968, J. Adv. Enzyme Reg. 7:149; Holland et al., 1978, Biotechnology 17:4900; and Holland et al., 1981, J. Biol. Chem. 256:1385). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., 1980, J. Biol. Chem. 255:2073) and those for other glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other promoters that have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization (Holland, supra).

Terminator sequences may also be used to enhance expression when placed at the 3' end of the coding sequence. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. Any vector containing a yeast-compatible promoter, origin of replication, and

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other control sequences is suitable for use in constructing yeast expression vectors for the thermostable DNA polymerases of the present invention.

The nucleotide sequences which code for the
5 thermostable DNA polymerases of the present invention can also be expressed in eucaryotic host cell cultures derived from multicellular organisms. See, for example, Tissue Culture, Academic Press, Cruz and Patterson, editors (1973). Useful host cell lines
10 include COS-7, COS-A2, CV-1, murine cells such as murine myelomas N51 and VERO, HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for
15 example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers et al., 1978, Nature 273:113), or other viral promoters such as those derived from polyoma, adenovirus 2, bovine papilloma virus (BPV), or avian sarcoma viruses, or
20 immunoglobulin promoters and heat shock promoters. A system for expressing DNA in mammalian systems using a BPV vector system is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. General aspects of
25 mammalian cell host system transformations have been described by Axel, U.S. Patent No. 4,399,216. "Enhancer" regions are also important in optimizing expression; these are, generally, sequences found upstream of the promoter region. Origins of
30 replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

Plant cells can also be used as hosts, and control sequences compatible with plant cells, such as the
35 nopaline synthase promoter and polyadenylation signal sequences (Depicker et al., 1982, J. Mol. Appl. Gen.

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1:561) are available. Expression systems employing insect cells utilizing the control systems provided by baculovirus vectors have also been described (Miller et al., 1986, Genetic Engineering (Setlow et al., eds., Plenum Publishing) 8:277-297). Insect cell-based expression can be accomplished in Spodoptera frugiperda. These systems can also be used to produce recombinant thermostable polymerases of the present invention.

10 Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, 1972, Proc. Natl. Acad. Sci. USA 69:2110 is used for procaryotes or other
15 cells that contain substantial cell wall barriers. Infection with Agrobacterium tumefaciens (Shaw et al., 1983, Gene 23:315) is used for certain plant cells. For mammalian cells, the calcium phosphate precipitation method of Graham and van der Eb, 1978,
20 Virology 52:546 is preferred. Transformations into yeast are carried out according to the method of Van Solingen et al., 1977, J. Bact. 130:946 and Hsiao et al., 1979, Proc. Natl. Acad. Sci. USA 76:3829.

Once the desired thermostable DNA polymerase with
25 altered 5' to 3' exonuclease activity has been expressed in a recombinant host cell, purification of the protein may be desired. Although a variety of purification procedures can be used to purify the recombinant thermostable polymerases of the invention,
30 fewer steps may be necessary to yield an enzyme preparation of equal purity. Because E. coli host proteins are heat-sensitive, the recombinant thermostable DNA polymerases of the invention can be substantially enriched by heat inactivating the crude
35 lysate. This step is done in the presence of a sufficient amount of salt (typically 0.2-0.3 M ammonium

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sulfate) to ensure dissociation of the thermostable DNA polymerase from the host DNA and to reduce ionic interactions of thermostable DNA polymerase with other cell lysate proteins.

5 In addition, the presence of 0.3 M ammonium sulfate promotes hydrophobic interaction with a phenyl sepharose column. Hydrophobic interaction chromatography is a separation technique in which substances are separated on the basis of differing
10 strengths of hydrophobic interaction with an uncharged bed material containing hydrophobic groups. Typically, the column is first equilibrated under conditions favorable to hydrophobic binding, such as high ionic strength. A descending salt gradient may then be used
15 to elute the sample.

According to the invention, an aqueous mixture (containing the recombinant thermostable DNA polymerase with altered 5' to 3' exonuclease activity) is loaded onto a column containing a relatively strong
20 hydrophobic gel such as phenyl sepharose (manufactured by Pharmacia) or Phenyl TSK (manufactured by Toyo Soda). To promote hydrophobic interaction with a phenyl sepharose column, a solvent is used that contains, for example, greater than or equal to 0.3 M
25 ammonium sulfate, with 0.3 M being preferred, or greater than or equal to 0.5 M NaCl. The column and the sample are adjusted to 0.3 M ammonium sulfate in 50 mM Tris (pH 7.5) and 1.0 mM EDTA ("TE") buffer that also contains 0.5 mM DTT, and the sample is applied to
30 the column. The column is washed with the 0.3 M ammonium sulfate buffer. The enzyme may then be eluted with solvents that attenuate hydrophobic interactions, such as decreasing salt gradients, ethylene or propylene glycol, or urea.

35 For long-term stability, the thermostable DNA polymerase enzymes of the present invention can be

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stored in a buffer that contains one or more non-ionic polymeric detergents. Such detergents are generally those that have a molecular weight in the range of approximately 100 to 250,000 daltons, preferably about 5 4,000 to 200,000 daltons, and stabilize the enzyme at a pH of from about 3.5 to about 9.5, preferably from about 4 to 8.5. Examples of such detergents include those specified on pages 295-298 of McCutcheon's Emulsifiers & Detergents, North American edition 10 (1983), published by the McCutcheon Division of MC Publishing Co., 175 Rock Road, Glen Rock, NJ (USA) and copending Serial No. 387,003, filed July 28, 1989, each of which is incorporated herein by reference.

Preferably, the detergents are selected from the 15 group comprising ethoxylated fatty alcohol ethers and lauryl ethers, ethoxylated alkyl phenols, octylphenoxy polyethoxy ethanol compounds, modified oxyethylated and/or oxypropylated straight-chain alcohols, polyethylene glycol monooleate compounds, polysorbate 20 compounds, and phenolic fatty alcohol ethers. More particularly preferred are Tween 20, a polyoxyethylated (20) sorbitan monolaurate from ICI Americas Inc., Wilmington, DE, and Iconol NP-40, an ethoxylated alkyl phenol (nonyl) from BASF Wyandotte Corp., Parsippany, 25 NJ.

The thermostable enzymes of this invention may be used for any purpose in which such enzyme activity is necessary or desired.

DNA sequencing by the Sanger dideoxynucleotide 30 method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74:5463-5467) has undergone significant refinement in recent years, including the development of novel vectors (Yanisch-Perron et al., 1985, Gene 33:103-119), base analogs (Mills et al., 1979, Proc. Natl. Acad. Sci. USA 76:2232-2235, and Barr et al., 1986, 35 BioTechniques 4:428-432), enzymes (Tabor et al., 1987,

Proc. Natl. Acad. Sci. USA 84:4763-4771, and Innis, M.A. et al., 1988, Proc. Natl. Acad. Sci. USA 85:9436:9440), and instruments for partial automation of DNA sequence analysis (Smith et al., 1986, Nature 321:674-679; Prober et al., 1987, Science 238:336-341; and Ansorge et al., 1987, Nuc. Acids Res. 15:4593-4602). The basic dideoxy sequencing procedure involves (i) annealing an oligonucleotide primer to a suitable single or denatured double stranded DNA template; (ii) extending the primer with DNA polymerase in four separate reactions, each containing one α -labeled dNTP or ddNTP (alternatively, a labeled primer can be used), a mixture of unlabeled dNTPs, and one chain-terminating dideoxynucleotide-5'-triphosphate (ddNTP); (iii) resolving the four sets of reaction products on a high-resolution polyacrylamide-urea gel; and (iv) producing an autoradiographic image of the gel that can be examined to infer the DNA sequence. Alternatively, fluorescently labeled primers or nucleotides can be used to identify the reaction products. Known dideoxy sequencing methods utilize a DNA polymerase such as the Klenow fragment of E. coli DNA polymerase I, reverse transcriptase, Tag DNA polymerase, or a modified T7 DNA polymerase.

The introduction of commercial kits has vastly simplified the art, making DNA sequencing a routine technique for any laboratory. However, there is still a need in the art for sequencing protocols that work well with nucleic acids that contain secondary structure such as palindromic hairpin loops and with G+C-rich DNA. Single stranded DNAs can form secondary structure, such as a hairpin loop, that can seriously interfere with a dideoxy sequencing protocol, both through improper termination in the extension reaction, or in the case of an enzyme with 5' to 3' exonuclease activity, cleavage of the template strand at the

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juncture of the hairpin. Since high temperature destabilizes secondary structure, the ability to conduct the extension reaction at a high temperature, i.e., 70-75°C, with a thermostable DNA polymerase results in a significant improvement in the sequencing of DNA that contains such secondary structure. However, temperatures compatible with polymerase extension do not eliminate all secondary structure. A 5' to 3' exonuclease-deficient thermostable DNA polymerase would be a further improvement in the art, since the polymerase could synthesize through the hairpin in a strand displacement reaction, rather than cleaving the template, resulting in an improper termination, i.e., an extension run-off fragment.

As an alternative to basic dideoxy sequencing, cycle dideoxy sequencing is a linear, asymmetric amplification of target sequences in the presence of dideoxy chain terminators. A single cycle produces a family of extension products of all possible lengths. Following denaturation of the extension reaction product from the DNA template, multiple cycles of primer annealing and primer extension occur in the presence of dideoxy terminators. The process is distinct from PCR in that only one primer is used, the growth of the sequencing reaction products in each cycle is linear, and the amplification products are heterogeneous in length and do not serve as template for the next reaction. Cycle dideoxy sequencing is a technique providing advantages for laboratories using automated DNA sequencing instruments and for other high volume sequencing laboratories. It is possible to directly sequence genomic DNA, without cloning, due to the specificity of the technique and the increased amount of signal generated. Cycle sequencing protocols accommodate single and double stranded templates, including genomic, cloned, and PCR-amplified templates.

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Thermostable DNA polymerases have several advantages in cycle sequencing: they tolerate the stringent annealing temperatures which are required for specific hybridization of primer to genomic targets as well as tolerating the multiple cycles of high temperature denaturation which occur in each cycle. Performing the extension reaction at high temperatures, i.e., 70-75°C, results in a significant improvement in sequencing results with DNA that contains secondary structure, due to the destabilization of secondary structure. However, such temperatures will not eliminate all secondary structure. A 5' to 3' exonuclease-deficient thermostable DNA polymerase would be a further improvement in the art, since the polymerase could synthesize through the hairpin in a strand displacement reaction, rather than cleaving the template and creating an improper termination. Additionally, like PCR, cycle sequencing suffers from the phenomenon of product strand renaturation. In the case of a thermostable DNA polymerase possessing 5' to 3' exonuclease activity, extension of a primer into a double stranded region created by product strand renaturation will result in cleavage of the renatured complementary product strand. The cleaved strand will be shorter and thus appear as an improper termination. In addition, the correct, previously synthesized termination signal will be attenuated. A thermostable DNA polymerase deficient in 5' to 3' exonuclease activity will improve the art, in that such extension product fragments will not be formed. A variation of cycle sequencing, involves the simultaneous generation of sequencing ladders for each strand of a double stranded template while sustaining some degree of amplification (Ruano and Kidd, Proc. Natl. Acad. Sci. USA, 1991 88:2815-2819). This method of coupled amplification and sequencing would benefit in a similar

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fashion as stranded cycle sequencing from the use of a thermostable DNA polymerase deficient in 5' to 3' exonuclease activity.

In a particularly preferred embodiment, the enzymes 5 in which the 5' to 3' exonuclease activity has been reduced or eliminated catalyze the nucleic acid amplification reaction known as PCR, and as stated above, with the resultant effect of producing a better yield of desired product than is achieved with the 10 respective native enzymes which have greater amounts of the 5' to 3' exonuclease activity. Improved yields are the result of the inability to degrade previously synthesized product caused by 5' to 3' exonuclease activity. This process for amplifying nucleic acid 15 sequences is disclosed and claimed in U.S. Patent Nos. 4,683,202 and 4,865,188, each of which is incorporated herein by reference. The PCR nucleic acid amplification method involves amplifying at least one specific nucleic acid sequence contained in a nucleic 20 acid or a mixture of nucleic acids and in the most common embodiment, produces double-stranded DNA. Aside from improved yields, thermostable DNA polymerases with attenuated 5' to 3' exonuclease activity exhibit an improved ability to generate longer PCR products, an 25 improved ability to produce products from G+C-rich templates and an improved ability to generate PCR products and DNA sequencing ladders from templates with a high degree of secondary structure.

For ease of discussion, the protocol set forth 30 below assumes that the specific sequence to be amplified is contained in a double-stranded nucleic acid. However, the process is equally useful in amplifying single-stranded nucleic acid, such as mRNA, although in the preferred embodiment the ultimate 35 product is still double-stranded DNA. In the amplification of a single-stranded nucleic acid, the

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first step involves the synthesis of a complementary strand (one of the two amplification primers can be used for this purpose), and the succeeding steps proceed as in the double-stranded amplification process 5 described below.

This amplification process comprises the steps of:

(a) contacting each nucleic acid strand with four 10 different nucleoside triphosphates and two oligonucleotide primers for each specific sequence being amplified, wherein each primer is selected to be substantially complementary to the different strands of the specific sequence, such that the extension product 15 synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, said contacting being at a temperature that allows hybridization of each primer to a complementary nucleic 20 acid strand;

(b) contacting each nucleic acid strand, at the same time as or after step (a), with a thermostable DNA polymerase of the present invention that enables combination of the nucleoside triphosphates to form 25 primer extension products complementary to each strand of the specific nucleic acid sequence;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each 30 different sequence being amplified, an extension product of each primer that is complementary to each nucleic acid strand template, but not so high as to separate each extension product from the complementary strand template;

35 (d) heating the mixture from step (c) for an effective time and at an effective temperature to

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separate the primer extension products from the templates on which they were synthesized to produce single-stranded molecules but not so high as to denature irreversibly the enzyme;

5 (e) cooling the mixture from step (d) for an effective time and to an effective temperature to promote hybridization of a primer to each of the single-stranded molecules produced in step (d); and

(f) maintaining the mixture from step (e) at an
10 effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer that is complementary to each nucleic acid template produced in step (d) but not so
15 high as to separate each extension product from the complementary strand template. The effective times and temperatures in steps (e) and (f) may coincide, so that steps (e) and (f) can be carried out simultaneously. Steps (d)-(f) are repeated until the desired level of
20 amplification is obtained.

The amplification method is useful not only for producing large amounts of a specific nucleic acid sequence of known sequence but also for producing nucleic acid sequences that are known to exist but are
25 not completely specified. One need know only a sufficient number of bases at both ends of the sequence in sufficient detail so that two oligonucleotide primers can be prepared that will hybridize to different strands of the desired sequence at relative
30 positions along the sequence such that an extension product synthesized from one primer, when separated from the template (complement), can serve as a template for extension of the other primer into a nucleic acid sequence of defined length. The greater the knowledge
35 about the bases at both ends of the sequence, the greater can be the specificity of the primers for the

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target nucleic acid sequence and the efficiency of the process and specificity of the reaction.

In any case, an initial copy of the sequence to be amplified must be available, although the sequence need
5 not be pure or a discrete molecule. In general, the amplification process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given that (a) the ends
10 of the required sequence are known in sufficient detail that oligonucleotides can be synthesized that will hybridize to them and (b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete
15 nucleic acid duplex with termini corresponding to the 5' ends of the specific primers employed.

Any nucleic acid sequence, in purified or nonpurified form, can be utilized as the starting nucleic acid(s), provided it contains or is suspected
20 to contain the specific nucleic acid sequence one desires to amplify. The nucleic acid to be amplified can be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including
25 bacteria, yeast, viruses, organelles, and higher organisms such as plants and animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi, or amniotic cells by a variety of techniques. See, e.g., Maniatis et al., 1982,
30 Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) pp. 280-281. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single-stranded or double-stranded. In
35 addition, a DNA-RNA hybrid that contains one strand of each may be utilized. A mixture of any of these

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nucleic acids can also be employed as can nucleic acids produced from a previous amplification reaction (using the same or different primers). The specific nucleic acid sequence to be amplified can be only a fraction of a large molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid.

The sequence to be amplified need not be present initially in a pure form; the sequence can be a minor fraction of a complex mixture, such as a portion of the β -globin gene contained in whole human DNA (as exemplified in Saiki *et al.*, 1985, *Science* 230:1530-1534) or a portion of a nucleic acid sequence due to a particular microorganism, which organism might constitute only a very minor fraction of a particular biological sample. The cells can be directly used in the amplification process after suspension in hypotonic buffer and heat treatment at about 90°C-100°C until cell lysis and dispersion of intracellular components occur (generally 1 to 15 minutes). After the heating step, the amplification reagents may be added directly to the lysed cells. The starting nucleic acid sequence can contain more than one desired specific nucleic acid sequence. The amplification process is useful not only for producing large amounts of one specific nucleic acid sequence but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

Primers play a key role in the PCR process. The word "primer" as used in describing the amplification process can refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified or where one employs the degenerate primer process described in PCT Application No. 91/05753, filed August 13, 1991. For instance, in

the case where a nucleic acid sequence is inferred from protein sequence information, a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code can be used for each strand. One primer from this collection will be sufficiently homologous with a portion of the desired sequence to be amplified so as to be useful for amplification.

In addition, more than one specific nucleic acid sequence can be amplified from the first nucleic acid or mixture of nucleic acids, so long as the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences, and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

A sequence within a given sequence can be amplified after a given number of amplification cycles to obtain greater specificity in the reaction by adding, after at least one cycle of amplification, a set of primers that are complementary to internal sequences (i.e., sequences that are not on the ends) of the sequence to be amplified. Such primers can be added at any stage and will provide a shorter amplified fragment. Alternatively, a longer fragment can be prepared by using primers with non-complementary ends but having some overlap with the primers previously utilized in the amplification.

Primers also play a key role when the amplification process is used for in vitro mutagenesis. The product of an amplification reaction where the primers employed are not exactly complementary to the original template

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will contain the sequence of the primer rather than the template, so introducing an in vitro mutation. In further cycles, this mutation will be amplified with an undiminished efficiency because no further mispaired priming is required. The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers to induce further sequence changes. In this way, a series of mutated sequences can gradually be produced wherein each new addition to the series differs from the last in a minor way, but from the original DNA source sequence in an increasingly major way.

Because the primer can contain as part of its sequence a non-complementary sequence, provided that a sufficient amount of the primer contains a sequence that is complementary to the strand to be amplified, many other advantages can be realized. For example, a nucleotide sequence that is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5' end of one or both of the primers and so appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

Oligonucleotide primers can be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and can be synthesized as described by Beaucage et al., 1981, Tetrahedron Letters 22:1859-1862. One method for synthesizing

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oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. One can also use a primer that has been isolated from a biological source (such as a restriction endonuclease digest).

5 No matter what primers are used, however, the reaction mixture must contain a template for PCR to occur, because the specific nucleic acid sequence is produced by using a nucleic acid containing that sequence as a template. The first step involves
10 contacting each nucleic acid strand with four different nucleoside triphosphates and two oligonucleotide primers for each specific nucleic acid sequence being amplified or detected. If the nucleic acids to be amplified or detected are DNA, then the nucleoside
15 triphosphates are usually dATP, dCTP, dGTP, and dTTP, although various nucleotide derivatives can also be used in the process. For example, when using PCR for the detection of a known sequence in a sample of unknown sequences, dTTP is often replaced by dUTP in
20 order to reduce contamination between samples as taught in PCT Application No. 91/05210 filed July 23, 1991, incorporated herein by reference.

The concentration of nucleoside triphosphates can vary widely. Typically, the concentration is 50 to 200
25 μM in each dNTP in the buffer for amplification, and MgCl_2 is present in the buffer in an amount of 1 to 3 mM to activate the polymerase and increase the specificity of the reaction. However, dNTP concentrations of 1 to 20 μM may be preferred for some
30 applications, such as DNA sequencing or generating radiolabeled probes at high specific activity.

The nucleic acid strands of the target nucleic acid serve as templates for the synthesis of additional nucleic acid strands, which are extension products of
35 the primers. This synthesis can be performed using any suitable method, but generally occurs in a buffered

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aqueous solution, preferably at a pH of 7 to 9, most preferably about 8. To facilitate synthesis, a molar excess of the two oligonucleotide primers is added to the buffer containing the template strands. As a practical matter, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process. Accordingly, primer:template ratios of at least 1000:1 or higher are generally employed for cloned DNA templates, and primer: template ratios of about 10^8 :1 or higher are generally employed for amplification from complex genomic samples.

The mixture of template, primers, and nucleoside triphosphates is then treated according to whether the nucleic acids being amplified or detected are double- or single-stranded. If the nucleic acids are single-stranded, then no denaturation step need be employed prior to the first extension cycle, and the reaction mixture is held at a temperature that promotes hybridization of the primer to its complementary target (template) sequence. Such temperature is generally from about 35°C to 65°C or more, preferably about 37°C to 60°C for an effective time, generally from a few seconds to five minutes, preferably from 30 seconds to one minute. A hybridization temperature of 35°C to 70°C may be used for 5' to 3' exonuclease mutant thermostable DNA polymerases. Primers that are 15 nucleotides or longer in length are used to increase the specificity of primer hybridization. Shorter primers require lower hybridization temperatures.

The complement to the original single-stranded nucleic acids can be synthesized by adding the thermostable DNA polymerase of the present invention in

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the presence of the appropriate buffer, dNTPs, and one or more oligonucleotide primers. If an appropriate single primer is added, the primer extension product will be complementary to the single-stranded nucleic acid and will be hybridized with the nucleic acid strand in a duplex of strands of equal or unequal length (depending on where the primer hybridizes to the template), which may then be separated into single strands as described above to produce two single, separated, complementary strands. A second primer would then be added so that subsequent cycles of primer extension would occur using both the original single-stranded nucleic acid and the extension product of the first primer as templates. Alternatively, two or more appropriate primers (one of which will prime synthesis using the extension product of the other primer as a template) can be added to the single-stranded nucleic acid and the reaction carried out.

20 If the nucleic acid contains two strands, as in the case of amplification of a double-stranded target or second-cycle amplification of a single-stranded target, the strands of nucleic acid must be separated before the primers are hybridized. This strand separation can be accomplished by any suitable denaturing method, including physical, chemical or enzymatic means. One preferred physical method of separating the strands of the nucleic acid involves heating the nucleic acid until complete (>99%) denaturation occurs. Typical heat denaturation involves temperatures ranging from about 80°C to 105°C for times generally ranging from about a few seconds to minutes, depending on the composition and size of the nucleic acid. Preferably, the effective denaturing temperature is 90°C-100°C for a few seconds to 1 minute. Strand separation may also be induced by an enzyme from the class of enzymes known

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as helicases or the enzyme RecA, which has helicase activity and in the presence of ATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases 5 are described by Kuhn Hoffmann-Berling, 1978, CSH-Quantitative Biology 43:63, and techniques for using RecA are reviewed in Radding, 1982, Ann. Rev. Genetics 16:405-437. The denaturation produces two separated complementary strands of equal or unequal 10 length.

If the double-stranded nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature that promotes hybridization of each primer to the complementary target (template) sequence. This 15 temperature is usually from about 35°C to 65°C or more, depending on reagents, preferably 37°C to 60°C. The hybridization temperature is maintained for an effective time, generally a few seconds to minutes, and preferably 10 seconds to 1 minute. In practical terms, 20 the temperature is simply lowered from about 95°C to as low as 37°C, and hybridization occurs at a temperature within this range.

Whether the nucleic acid is single- or double-stranded, the thermostable DNA polymerase of the 25 present invention can be added prior to or during the denaturation step or when the temperature is being reduced to or is in the range for promoting hybridization. Although the thermostability of the polymerases of the invention allows one to add such 30 polymerases to the reaction mixture at any time, one can substantially inhibit non-specific amplification by adding the polymerase to the reaction mixture at a point in time when the mixture will not be cooled below the stringent hybridization temperature. After 35 hybridization, the reaction mixture is then heated to or maintained at a temperature at which the activity of

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the enzyme is promoted or optimized, i.e., a temperature sufficient to increase the activity of the enzyme in facilitating synthesis of the primer extension products from the hybridized primer and 5 template. The temperature must actually be sufficient to synthesize an extension product of each primer that is complementary to each nucleic acid template, but must not be so high as to denature each extension product from its complementary template (i.e., the 10 temperature is generally less than about 80°C to 90°C).

Depending on the nucleic acid(s) employed, the typical temperature effective for this synthesis reaction generally ranges from about 40°C to 80°C, preferably 50°C to 75°C. The temperature more 15 preferably ranges from about 65°C to 75°C for the thermostable DNA polymerases of the present invention. The period of time required for this synthesis may range from about 10 seconds to several minutes or more, depending mainly on the temperature, the length of the 20 nucleic acid, the enzyme, and the complexity of the nucleic acid mixture. The extension time is usually about 30 seconds to a few minutes. If the nucleic acid is longer, a longer time period is generally required for complementary strand synthesis.

25 The newly synthesized strand and the complement nucleic acid strand form a double-stranded molecule that is used in the succeeding steps of the amplification process. In the next step, the strands of the double-stranded molecule are separated by heat 30 denaturation at a temperature and for a time effective to denature the molecule, but not at a temperature and for a period so long that the thermostable enzyme is completely and irreversibly denatured or inactivated. After this denaturation of template, the temperature is 35 decreased to a level that promotes hybridization of the

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primer to the complementary single-stranded molecule (template) produced from the previous step, as described above.

After this hybridization step, or concurrently with the hybridization step, the temperature is adjusted to a temperature that is effective to promote the activity of the thermostable enzyme to enable synthesis of a primer extension product using as a template both the newly synthesized and the original strands. The temperature again must not be so high as to separate (denature) the extension product from its template, as described above. Hybridization may occur during this step, so that the previous step of cooling after denaturation is not required. In such a case, using simultaneous steps, the preferred temperature range is 50°C to 70°C.

The heating and cooling steps involved in one cycle of strand separation, hybridization, and extension product synthesis can be repeated as many times as needed to produce the desired quantity of the specific nucleic acid sequence. The only limitation is the amount of the primers, thermostable enzyme, and nucleoside triphosphates present. Usually, from 15 to 30 cycles are completed. For diagnostic detection of amplified DNA, the number of cycles will depend on the nature of the sample, the initial target concentration in the sample and the sensitivity of the detection process used after amplification. For a given sensitivity of detection, fewer cycles will be required if the sample being amplified is pure and the initial target concentration is high. If the sample is a complex mixture of nucleic acids and the initial target concentration is low, more cycles will be required to amplify the signal sufficiently for detection. For general amplification and detection, the process is repeated about 15 times. When amplification is used to

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generate sequences to be detected with labeled sequence-specific probes and when human genomic DNA is the target of amplification, the process is repeated 15 to 30 times to amplify the sequence sufficiently so 5 that a clearly detectable signal is produced, i.e., so that background noise does not interfere with detection.

No additional nucleotides, primers, or thermostable enzyme need be added after the initial addition, provided that no key reagent has been exhausted and 10 that the enzyme has not become denatured or irreversibly inactivated, in which case additional polymerase or other reagent would have to be added for the reaction to continue. After the appropriate number of cycles has been completed to produce the desired 15 amount of the specific nucleic acid sequence, the reaction can be halted in the usual manner, e.g., by inactivating the enzyme by adding EDTA, phenol, SDS, or CHCl_3 or by separating the components of the reaction.

The amplification process can be conducted 20 continuously. In one embodiment of an automated process, the reaction mixture can be temperature cycled such that the temperature is programmed to be controlled at a certain level for a certain time. One such instrument for this purpose is the automated 25 machine for handling the amplification reaction developed and marketed by Perkin-Elmer Cetus Instruments. Detailed instructions for carrying out PCR with the instrument are available upon purchase of the instrument.

30 The thermostable DNA polymerases of the present invention with altered 5' to 3' exonuclease activity are very useful in the diverse processes in which amplification of a nucleic acid sequence by PCR is useful. The amplification method may be utilized to 35 clone a particular nucleic acid sequence for insertion into a suitable expression vector, as described in U.S.

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Patent No. 4,800,159. The vector may be used to transform an appropriate host organism to produce the gene product of the sequence by standard methods of recombinant DNA technology. Such cloning may involve
5 direct ligation into a vector using blunt-end ligation, or use of restriction enzymes to cleave at sites contained within the primers. Other processes suitable for the thermostable DNA polymerases of the present invention include those described in U.S. Patent Nos.
10 4,683,195 and 4,683,202 and European Patent Publication Nos. 229,701; 237,362; and 258,017; these patents and publications are incorporated herein by reference. In addition, the present enzyme is useful in asymmetric PCR (see Gyllensten and Erlich, 1988, Proc. Natl. Acad. Sci. USA 85:7652-7656, incorporated herein by
15 reference); inverse PCR (Ochman et al., 1988, Genetics 120:621, incorporated herein by reference); and for DNA sequencing (see Innis et al., 1988, Proc. Natl. Acad. Sci. USA 85:9436-9440, and McConlogue et al., 1988,
20 Nuc. Acids Res. 16(20):9869), random amplification of cDNA ends (RACE), random priming PCR which is used to amplify a series of DNA fragments, and PCR processes with single sided specificity such as anchor PCR and ligation-mediated anchor PCR as described by Loh, E. in
25 METHODS: A Companion to Methods in Enzymology (1991) 2: pp. 11-19.

An additional process in which a 5' to 3' exonuclease deficient thermostable DNA polymerase would be useful is a process referred to as polymerase ligase
30 chain reaction (PLCR). As its name suggests, this process combines features of PCR with features of ligase chain reaction (LCR).

PLCR was developed in part as a technique to increase the specificity of allele-specific PCR in
35 which the low concentrations of dNTPs utilized (~1 μ M) limited the extent of amplification. In PLCR, DNA is

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denatured and four complementary, but not adjacent, oligonucleotide primers are added with dNTPs, a thermostable DNA polymerase and a thermostable ligase.

The primers anneal to target DNA in a non-adjacent fashion and the thermostable DNA polymerase causes the addition of appropriate dNTPs to the 3' end of the downstream primer to fill the gap between the non-adjacent primers and thus render the primers adjacent. The thermostable ligase will then ligate the two adjacent oligonucleotide primers.

However, the presence of 5' to 3' exonuclease activity in the thermostable DNA polymerase significantly decreases the probability of closing the gap between the two primers because such activity causes the excision of nucleotides or small oligonucleotides from the 5' end of the downstream primer thus preventing ligation of the primers. Therefore, a thermostable DNA polymerase with attenuated or eliminated 5' to 3' exonuclease activity would be particularly useful in PLCR.

Briefly, the thermostable DNA polymerases of the present invention which have been mutated to have reduced, attenuated or eliminated 5' to 3' exonuclease activity are useful for the same procedures and techniques as their respective non-mutated polymerases except for procedures and techniques which require 5' to 3' exonuclease activity such as the homogeneous assay technique discussed below. Moreover, the mutated DNA polymerases of the present invention will oftentimes result in more efficient performance of the procedures and techniques due to the reduction or elimination of the inherent 5' to 3' exonuclease activity.

Specific thermostable DNA polymerases with attenuated 5' to 3' exonuclease activity include the following mutated forms of Taq, Tma, Tsps17, TZ05, Tth

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and Taf DNA polymerases. In the table below, and throughout the specification, deletion mutations are inclusive of the numbered nucleotides or amino acids which define the deletion.

5	<u>DNA</u> <u>Polymerase</u>	<u>Mutation</u>	<u>Mutant</u> <u>Designation</u>
10	<u>Taq</u>	G(137) to A in nucleotide SEQ ID NO:1	pRDA3-2
		Gly (46) to Asp in amino acid SEQ ID NO:2	ASP46 <u>Taq</u>
15		Deletion of nucleotides 4-228 of nucleotide SEQ ID NO:1	pTAQd2-76
20		Deletion of amino acids 2-76 of amino acid SEQ ID NO:2	MET-ALA 77 <u>Taq</u>
25		Deletion of nucleotides 4-138 of nucleotide SEQ ID NO:1	pTAQd2-46
		Deletion of amino acids 2-46 of amino acid SEQ ID NO:2	MET-PHE 47 <u>Taq</u>
30		Deletion of nucleotides 4-462 of nucleotide SEQ ID NO:1	pTAQd2-155
35		Deletion of amino acids 2-154 of amino acid SEQ ID NO:2	MET-VAL 155 <u>Taq</u>
40		Deletion of nucleotides 4-606 of nucleotide SEQ ID NO:1	pTAQd2-202
45		Deletion of amino acids 2-202 of amino acid SEQ ID NO:2	MET-THR 203 <u>Taq</u>
50		Deletion of nucleotides 4-867 of nucleotide SEQ ID NO:1	pLSG8

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	Deletion of amino acids 2-289 of amino acid SEQ ID NO:2	MET-SER 290 <u>Tag</u> (Stoffel fragment)
5	<u>Tma</u>	
	G(110) to A in nucleotide SEQ ID NO:3	
10	Gly (37) to Asp in amino acid SEQ ID NO:4	ASP37 <u>Tma</u>
	Deletion of nucleotides 4-131 of nucleotide SEQ ID NO:3	pTMAd2-37
15		
	Deletion of amino acids 2-37 of amino acid SEQ ID NO:4	MET-VAL 38 <u>Tma</u>
20	Deletion of nucleotides 4-60 of nucleotide SEQ ID NO:3	pTMAd2-20
	Deletion of amino acids 2-20 of amino acid SEQ ID NO:4	MET-ASP 21 <u>Tma</u>
25		
	Deletion of nucleotides 4-219 of nucleotide SEQ ID NO:3	pTMAd2-73
30		
	Deletion of amino acids 2-73 amino acid SEQ ID NO: 4	MET-GLU 74 <u>Tma</u>
35		
	Deletion of nucleotides 1-417 of nucleotide SEQ ID NO:3	pTMA16
40	Deletion of amino acids 1-139 of amino acid SEQ ID NO:4	MET 140 <u>Tma</u>
	Deletion of nucleotides 1-849 of nucleotide SEQ ID NO:3	pTMA15
45		
	Deletion of amino acids 1-283 of amino acid SEQ ID NO:4	MET 284 <u>Tma</u>
50		
	<u>Tsps17</u>	
	G(128) to A in nucleotide SEQ ID NO:5	

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	Gly (43) to Asp in amino acid SEQ ID NO:6	ASP43 <u>Tsps17</u>
5	Deletion of nucleotides 4-129 of nucleotide SEQ ID NO:5	pSPSd2-43
10	Deletion of amino acids 2-43 of amino acid SEQ ID NO:6	MET-PHE 44 <u>Tsps17</u>
15	Deletion of nucleotides 4-219 of nucleotide SEQ ID NO:5	pSPSd2-73
20	Deletion of amino acids 2-73 of amino acid SEQ ID NO:6	MET-ALA 74 <u>Tsps17</u>
25	Deletion of nucleotides 4-453 of nucleotide SEQ ID NO:5	pSPSd2-151
30	Deletion of amino acids 2-151 of amino acid SEQ ID NO:6	MET-LEU 152 <u>Tsps17</u>
35	Deletion of nucleotides 4-597 of nucleotide SEQ ID NO:5	pSPSd2-199
40	Deletion of amino acids 2-199 of amino acid SEQ ID NO:6	MET-THR 200 <u>Tsps17</u>
45	Deletion of nucleotides 4-861 of nucleotide SEQ ID NO:5	pSPSA288
	Deletion of amino acids 2-287 of amino acid SEQ ID NO:6	MET-ALA 288 <u>Tsps 17</u>
	<u>TZ05</u> G(137) to A in nucleotide SEQ ID NO:7	
50	Gly (46) to Asp in amino acid SEQ ID NO:8	ASP46 <u>TZ05</u>
	Deletion of nucleotides 4-138 of nucleotide SEQ ID NO:7	pZ05d2-46

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	Deletion of amino acids 2-46 of amino acid SEQ ID NO:8	MET-PHE 47 <u>TZ05</u>
5	Deletion of nucleotides 4-231 of nucleotide SEQ ID NO:7	pZ05d2-77
10	Deletion of amino acids 2-77 of amino acid SEQ ID NO:8	MET-ALA 78 <u>TZ05</u>
15	Deletion of nucleotides 4-475 of nucleotide SEQ ID NO:7	pZ05d2-155
20	Deletion of amino acids 2-155 of amino acid SEQ ID NO:8	MET-VAL 156 <u>TZ05</u>
	Deletion of nucleotides 4-609 of nucleotide SEQ ID NO:7	pZ05d2-203
25	Deletion of amino acids 2-203 of amino acid SEQ ID NO:8	MET-THR 204 <u>TZ05</u>
30	Deletion of nucleotides 4-873 of nucleotide SEQ ID NO:7	pZ05A292
35	Deletion of amino acids 2-291 of amino acid SEQ ID NO:8	MET-ALA 292 <u>TZ05</u>
<u>Tth</u>	G(137) to A in nucleotide SEQ ID NO:9	
40	Gly (46) to Asp in amino acid SEQ ID NO:10	ASP46 <u>Tth</u>
45	Deletion of nucleotides 4-138 of nucleotide SEQ ID NO:9	pTTHd2-46
50	Deletion of amino acids 2-46 of amino acid SEQ ID NO:10	MET-PHE 47 <u>Tth</u>
	Deletion of nucleotides 4-231 of nucleotide SEQ ID NO:9	pTTHd2-77

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	Deletion of amino acids 2-77 of amino acid SEQ ID NO:10	MET-ALA 78 <u>Tth</u>
5	Deletion of nucleotides 4-465 of nucleotide SEQ ID NO:9	pTTHd2-155
10	Deletion of amino acids 2-155 of amino acid SEQ ID NO:10	MET-VAL 156 <u>Tth</u>
15	Deletion of nucleotides 4-609 of nucleotide SEQ ID NO:9	pTTHd2-203
20	Deletion of amino acids 2-203 of amino acid SEQ ID NO:10	MET-THR 204 <u>Tth</u>
	Deletion of nucleotides 4-873 of nucleotide SEQ ID NO:9	pTTHA292
25	Deletion of amino acids 2-291 of amino acid SEQ ID NO:10	MET-ALA 292 <u>Tth</u>
30	<u>Taf</u> G(110) to A and A(111) to T in nucleotide SEQ ID NO:11	
35	Gly (37) to Asp in amino acid SEQ ID NO:12	ASP37 <u>Taf</u>
	Deletion of nucleotides 4-111 of nucleotide SEQ ID NO:11	pTAFd2-37
40	Deletion of amino acids 2-37 of amino acid SEQ ID NO:12	MET-LEU 38 <u>Taf</u>
45	Deletion of nucleotides 4-279 of nucleotide SEQ ID NO:11	pTAF09
50	Deletion of amino acids 2-93 amino acid SEQ ID NO:12	MET-TYR 94 <u>Taf</u>

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	Deletion of nucleotides 4-417 of nucleotide SEQ ID NO:11	pTAF11
5	Deletion of amino acids 2-139 of amino acid SEQ ID NO:12	MET-GLU 140 <u>Taf</u>
10	Deletion of nucleotides 4-609 of nucleotide SEQ ID NO:11	pTAFd2-203
15	Deletion of amino acids 2-203 of amino acid SEQ ID NO:12	MET-THR 204 <u>Taf</u>
20	Deletion of nucleotides 4-852 of nucleotide SEQ ID NO:11	pTAFI285
	Deletion of amino acids 2-284 of amino acid SEQ ID NO:12	MET-ILE 285 <u>Taf</u>
25	<u>Thermostable DNA Polymerases With Enhanced 5' to 3' Exonuclease Activity</u>	

Another aspect of the present invention involves
 30 the generation of thermostable DNA polymerases which
 exhibit enhanced or increased 5' to 3' exonuclease
 activity over that of their respective native
 polymerases. The thermostable DNA polymerases of the
 present invention which have increased or enhanced 5'
 35 to 3' exonuclease activity are particularly useful in
 the homogeneous assay system described in PCT
 application No. 91/05571 filed August 6, 1991, which is
 incorporated herein by reference. Briefly, this system
 is a process for the detection of a target amino acid
 40 sequence in a sample comprising:

(a) contacting a sample comprising single-stranded
 nucleic acids with an oligonucleotide containing a
 sequence complementary to a region of the target
 45 nucleic acid and a labeled oligonucleotide containing a

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- sequence complementary to a second region of the same target nucleic acid strand, but not including the nucleic acid sequence defined by the first oligonucleotide, to create a mixture of duplexes during hybridization conditions, wherein the duplexes comprise the target nucleic acid annealed to the first oligonucleotide and to the labeled oligonucleotide such that the 3' end of the first oligonucleotide is adjacent to the 5' end of the labeled oligonucleotide;
- 10 (b) maintaining the mixture of step (a) with a template-dependent nucleic acid polymerase having a 5' to 3' nuclease activity under conditions sufficient to permit the 5' to 3' nuclease activity of the polymerase to cleave the annealed, labeled oligonucleotide and
- 15 release labeled fragments; and
- (c) detecting and/or measuring the release of labeled fragments.

This homogeneous assay system is one which generates signal while the target sequence is amplified, thus, minimizing the post-amplification handling of the amplified product which is common to other assay systems. Furthermore, a particularly preferred use of the thermostable DNA polymerases with increased 5' to 3' exonuclease activity is in a homogeneous assay system which utilizes PCR technology. This particular assay system involves:

- (a) providing to a PCR assay containing said sample, at least one labeled oligonucleotide containing a sequence complementary to a region of the target nucleic acid, wherein said labeled oligonucleotide anneals within the target nucleic acid sequence bounded by the oligonucleotide primers of step (b);
- 30 (b) providing a set of oligonucleotide primers, wherein a first primer contains a sequence

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complementary to a region in one strand of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand, and a second primer contains a sequence complementary to a region in a second strand
5 of the target nucleic acid sequence and primes the synthesis of a complementary DNA strand; and wherein each oligonucleotide primer is selected to anneal to its complementary template upstream of any labeled oligonucleotide annealed to the same nucleic acid
10 strand;

(c) amplifying the target nucleic acid sequence employing a nucleic acid polymerase having 5' to 3' nuclease activity as a template-dependent polymerizing agent under conditions which are permissive for PCR
15 cycling steps of (i) annealing of primers and labeled oligonucleotide to a template nucleic acid sequence contained within the target region, and (ii) extending the primer, wherein said nucleic acid polymerase synthesizes a primer extension product while the 5' to
20 3' nuclease activity of the nucleic acid polymerase simultaneously releases labeled fragments from the annealed duplexes comprising labeled oligonucleotide and its complementary template nucleic acid sequences, thereby creating detectable labeled fragments; and
25 (d) detecting and/or measuring the release of labeled fragments to determine the presence or absence of target sequence in the sample.

The increased 5' to 3' exonuclease activity of the
30 thermostable DNA polymerases of the present invention when used in the homogeneous assay systems causes the cleavage of mononucleotides or small oligonucleotides from an oligonucleotide annealed to its larger, complementary polynucleotide. In order for cleavage to
35 occur efficiently, an upstream oligonucleotide must also be annealed to the same larger polynucleotide.

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The 3' end of this upstream oligonucleotide provides the initial binding site for the nucleic acid polymerase. As soon as the bound polymerase encounters the 5' end of the downstream oligonucleotide, the polymerase can cleave mononucleotides or small oligonucleotides therefrom.

The two oligonucleotides can be designed such that they anneal in close proximity on the complementary target nucleic acid such that binding of the nucleic acid polymerase to the 3' end of the upstream oligonucleotide automatically puts it in contact with the 5' end of the downstream oligonucleotide. This process, because polymerization is not required to bring the nucleic acid polymerase into position to accomplish the cleavage, is called "polymerization-independent cleavage".

Alternatively, if the two oligonucleotides anneal to more distantly spaced regions of the template nucleic acid target, polymerization must occur before the nucleic acid polymerase encounters the 5' end of the downstream oligonucleotide. As the polymerization continues, the polymerase progressively cleaves mononucleotides or small oligonucleotides from the 5' end of the downstream oligonucleotide. This cleaving continues until the remainder of the downstream oligonucleotide has been destabilized to the extent that it dissociates from the template molecule. This process is called "polymerization-dependent cleavage".

The attachment of label to the downstream oligonucleotide permits the detection of the cleaved mononucleotides and small oligonucleotides. Subsequently, any of several strategies may be employed to distinguish the uncleaved labelled oligonucleotide from the cleaved fragments thereof. In this manner, nucleic acid samples which contain sequences complementary to the upstream and downstream

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oligonucleotides can be identified. Stated differently, a labelled oligonucleotide is added concomittantly with the primer at the start of PCR, and the signal generated from hydrolysis of the labelled 5 nucleotide(s) of the probe provides a means for detection of the target sequence during its amplification.

In the homogeneous assay system process, a sample is provided which is suspected of containing the 10 particular oligonucleotide sequence of interest, the "target nucleic acid". The target nucleic acid contained in the sample may be first reverse transcribed into cDNA, if necessary, and then denatured, using any suitable denaturing method, 15 including physical, chemical, or enzymatic means, which are known to those of skill in the art. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves 20 temperatures ranging from about 80°C to about 105°C, for times ranging from a few seconds to minutes. As an alternative to denaturation, the target nucleic acid may exist in a single-stranded form in the sample, such as, for example, single-stranded RNA or DNA viruses.

25 The denatured nucleic acid strands are then incubated with preselected oligonucleotide primers and labeled oligonucleotide (also referred to herein as "probe") under hybridization conditions, conditions which enable the binding of the primers and probes to 30 the single nucleic acid strands. As known in the art, the primers are selected so that their relative positions along a duplex sequence are such that an extension product synthesized from one primer, when the extension product is separated from its template

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(complement), serves as a template for the extension of the other primer to yield a replicate chain of defined length.

Because the complementary strands are longer than 5 either the probe or primer, the strands have more points of contact and thus a greater chance of finding each other over any given period of time. A high molar excess of probe, plus the primer, helps tip the balance toward primer and probe annealing rather than template 10 reannealing.

The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length and composition of the primer will depend on many factors, 15 including temperature of the annealing reaction, source and composition of the primer, proximity of the probe annealing site to the primer annealing site, and ratio of primer:probe concentration. For example, depending on the complexity of the target sequence, the 20 oligonucleotide primer typically contains about 15-30 nucleotides, although a primer may contain more or fewer nucleotides. The primers must be sufficiently complementary to anneal to their respective strands selectively and form stable duplexes.

25 The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. The primers need not reflect the exact sequence of the template, but must be sufficiently complementary to hybridize 30 selectively to their respective strands. Non-complementary bases or longer sequences can be interspersed into the primer or located at the ends of the primer, provided the primer retains sufficient complementarity with a template strand to form a stable

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duplex therewith. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites.

In the practice of the homogeneous assay system, the labeled oligonucleotide probe must be first annealed to a complementary nucleic acid before the nucleic acid polymerase encounters this duplex region, thereby permitting the 5' to 3' exonuclease activity to cleave and release labeled oligonucleotide fragments.

To enhance the likelihood that the labeled oligonucleotide will have annealed to a complementary nucleic acid before primer extension polymerization reaches this duplex region, or before the polymerase attaches to the upstream oligonucleotide in the polymerization-independent process, a variety of techniques may be employed. For the polymerization-dependent process, one can position the probe so that the 5'-end of the probe is relatively far from the 3'-end of the primer, thereby giving the probe more time to anneal before primer extension blocks the probe binding site. Short primer molecules generally require lower temperatures to form sufficiently stable hybrid complexes with the target nucleic acid. Therefore, the labeled oligonucleotide can be designed to be longer than the primer so that the labeled oligonucleotide anneals preferentially to the target at higher temperatures relative to primer annealing.

One can also use primers and labeled oligonucleotides having differential thermal stability. For example, the nucleotide composition of the labeled oligonucleotide can be chosen to have greater G/C content and, consequently, greater thermal stability than the primer. In similar fashion, one can incorporate modified nucleotides into the probe, which

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modified nucleotides contain base analogs that form more stable base pairs than the bases that are typically present in naturally occurring nucleic acids.

Modifications of the probe that may facilitate probe binding prior to primer binding to maximize the efficiency of the present assay include the incorporation of positively charged or neutral phosphodiester linkages in the probe to decrease the repulsion of the polyanionic backbones of the probe and target (see Letsinger et al., 1988, J. Amer. Chem. Soc. 110:4470); the incorporation of alkylated or halogenated bases, such as 5-bromouridine, in the probe to increase base stacking; the incorporation of ribonucleotides into the probe to force the probe:target duplex into an "A" structure, which has increased base stacking; and the substitution of 2,6-diaminopurine (amino adenosine) for some or all of the adenosines in the probe. In preparing such modified probes of the invention, one should recognize that the rate limiting step of duplex formation is "nucleation", the formation of a single base pair, and therefore, altering the biophysical characteristic of a portion of the probe, for instance, only the 3' or 5' terminal portion, can suffice to achieve the desired result. In addition, because the 3' terminal portion of the probe (the 3' terminal 8 to 12 nucleotides) dissociates following exonuclease degradation of the 5' terminus by the polymerase, modifications of the 3' terminus can be made without concern about interference with polymerase/nuclease activity.

The thermocycling parameters can also be varied to take advantage of the differential thermal stability of the labeled oligonucleotide and primer. For example, following the denaturation step in thermocycling, an intermediate temperature may be introduced which is permissible for labeled oligonucleotide binding but not

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primer binding, and then the temperature is further reduced to permit primer annealing and extension. One should note, however, that probe cleavage need only occur in later cycles of the PCR process for suitable results. Thus, one could set up the reaction mixture so that even though primers initially bind preferentially to probes, primer concentration is reduced through primer extension so that, in later cycles, probes bind preferentially to primers.

10 To favor binding of the labeled oligonucleotide before the primer, a high molar excess of labeled oligonucleotide to primer concentration can also be used. In this embodiment, labeled oligonucleotide concentrations are typically in the range of about 2 to
15 20 times higher than the respective primer concentration, which is generally $0.5 - 5 \times 10^{-7}$ M. Those of skill recognize that oligonucleotide concentration, length, and base composition are each important factors that affect the T_m of any particular
20 oligonucleotide in a reaction mixture. Each of these factors can be manipulated to create a thermodynamic bias to favor probe annealing over primer annealing.

Of course, the homogeneous assay system can be applied to systems that do not involve amplification.
25 In fact, the present invention does not even require that polymerization occur. One advantage of the polymerization-independent process lies in the elimination of the need for amplification of the target sequence. In the absence of primer extension, the
30 target nucleic acid is substantially single-stranded. Provided the primer and labeled oligonucleotide are adjacently bound to the target nucleic acid, sequential rounds of oligonucleotide annealing and cleavage of labeled fragments can occur. Thus, a sufficient amount
35 of labeled fragments can be generated, making detection possible in the absence of polymerization. As would be

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appreciated by those skilled in the art, the signal generated during PCR amplification could be augmented by this polymerization-independent activity.

In addition to the homogeneous assay systems 5 described above, the thermostable DNA polymerases of the present invention with enhanced 5' to 3' exonuclease activity are also useful in other amplification systems, such as the transcription amplification system, in which one of the PCR primers 10 encodes a promoter that is used to make RNA copies of the target sequence. In similar fashion, the present invention can be used in a self-sustained sequence replication (3SR) system, in which a variety of enzymes are used to make RNA transcripts that are then used to 15 make DNA copies, all at a single temperature. By incorporating a polymerase with 5' to 3' exonuclease activity into a ligase chain reaction (LCR) system, together with appropriate oligonucleotides, one can also employ the present invention to detect LCR 20 products.

Also, just as 5' to 3' exonuclease deficient thermostable DNA polymerases are useful in PLCR, other thermostable DNA polymerases which have 5' to 3' exonuclease activity are also useful in PLCR under 25 different circumstances. Such is the case when the 5' tail of the downstream primer in PLCR is non-complementary to the target DNA. Such non-complementarity causes a forked structure where the 5' end of the upstream primer would normally anneal to 30 the target DNA.

Thermostable ligases cannot act on such forked structures. However, the presence of 5' to 3' exonuclease activity in the thermostable DNA polymerase will cause the excision of the forked 5' tail of the 35 upstream primer, thus permitting the ligase to act.

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The same processes and techniques which are described above as effective for preparing thermostable DNA polymerases with attenuated 5' to 3' exonuclease activity are also effective for preparing the 5 thermostable DNA polymerases with enhanced 5' to 3' exonuclease activity. As described above, these processes include such techniques as site-directed mutagenesis, deletion mutagenesis and "domain shuffling".

10 Of particular usefulness in preparing the thermostable DNA polymerases with enhanced 5' to 3' exonuclease activity is the "domain shuffling" technique described above. To briefly summarize, this technique involves the cleavage of a specific domain of 15 a polymerase which is recognized as coding for a very active 5' to 3' exonuclease activity of that polymerase, and then transferring that domain into the appropriate area of a second thermostable DNA polymerase gene which encodes a lower level or no 5' to 20 3' exonuclease activity. The desired domain may replace a domain which encodes an undesired property of the second thermostable DNA polymerase or be added to the nucleotide sequence of the second thermostable DNA polymerase.

25 A particular "domain shuffling" example is set forth above in which the Tma DNA polymerase coding sequence comprising codons about 291 through 484 is substituted for the Tag DNA polymerase I codons 289 through 422. This substitution yields a novel 30 thermostable DNA polymerase containing the 5' to 3' exonuclease domain of Tag DNA polymerase (codons 1-289), the 3' to 5' exonuclease domain of Tma DNA polymerase (codons 291-484) and the DNA polymerase domain of Tag DNA polymerase (codons 423-832). 35 However, those skilled in the art will recognize that other substitutions can be made in order to construct a

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thermostable DNA polymerase with certain desired characteristics such as enhanced 5' to 3' exonuclease activity.

The following examples are offered by way of illustration only and are by no means intended to limit the scope of the claimed invention. In these examples, all percentages are by weight if for solids and by volume if for liquids, unless otherwise noted, and all temperatures are given in degrees Celsius.

10

Example 1

Preparation of a 5' to 3' Exonuclease Mutant
of Tag DNA Polymerase by Random Mutagenesis
15 PCR of the Known 5' to 3' Exonuclease Domain

Preparation of Insert

Plasmid pLSG12 was used as a template for PCR.
20 This plasmid is a HindIII minus version of pLSG5 in which the Tag polymerase gene nucleotides 616 - 621 of SEQ ID NO:1 were changed from AAGCTT to AAGCTG. This change eliminated the HindIII recognition sequence within the Tag polymerase gene without altering encoded
25 protein sequence.

Using oligonucleotides MK61 (AGGACTACAACTGCCACACACC) (SEQ ID NO:21) and RA01 (CGAGGCGCGCCAGCCCCAGGAGATCTACC-AGCTCCTTG) (SEQ ID NO:22) as primers and pLSG12 as the template, PCR was conducted to amplify a 384 bp
30 fragment containing the ATG start of the Tag polymerase gene, as well as an additional 331 bp of coding sequence downstream of the ATG start codon.

A 100 μ l PCR was conducted for 25 cycles utilizing the following amounts of the following agents and
35 reactants:

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- 50 pmol of primer MK61 (SEQ ID NO:21);
- 50 pmol of primer RA01 (SEQ ID NO:22);
- 50 μ M of each dNTP;
- 10 mM Tris-HCl, pH 8.3;
- 5 50 mM KCl;
- 1.5 mM $MgCl_2$;
- 75.6 pg pLSG12;
- 2.5 units AmpliTaq DNA polymerase.

10 The PCR reaction mixture described was placed in a Perkin-Elmer Cetus Thermocycler and run through the following profile. The reaction mixture was first ramped up to 98°C over 1 minute and 45 seconds, and held at 98°C for 25 seconds. The reaction mixture was
15 then ramped down to 55°C over 45 seconds and held at that temperature for 20 seconds. Finally, the mixture was ramped up to 72°C over 45 seconds, and held at 72°C for 30 seconds. A final 5 minute extension occurred at 72°C.

20 The PCR product was then extracted with chloroform and precipitated with isopropanol using techniques which are well known in the art.

A 300 ng sample of the PCR product was digested with 20 U of HindIII (in 30 μ l reaction) for 2 hours at
25 37°C. Then, an additional digestion was made with 8 U of BssHII for an 2 hours at 50°C. This series of digestions yielded a 330 bp fragment for cloning.

A vector was prepared by digesting 5.3 μ g of pLSG12 with 20 U HindIII (in 40 μ l) for 2 hours at 37°C. This
30 digestion was followed by addition of 12 U of BssHII and incubation for 2 hours at 50°C.

The vector was dephosphorylated by treatment with CIAP (calf intestinal alkaline phosphatase), specifically 0.04 U CIAP for 30 minutes at 30°C. Then,

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4 μ l of 500 mM EGTA was added to the vector preparation to stop the reaction, and the phosphatase was inactivated by incubation at 65°C for 45 minutes.

225 ng of the phosphatased vector described above was ligated at a 1:1 molar ratio with 10 ng of the PCR-derived insert.

Then, DG116 cells were transformed with one fifth of the ligation mixture, and ampicillin-resistant transformants were selected at 30°C.

10 Appropriate colonies were grown overnight at 30°C to OD₆₀₀ 0.7. Cells containing the P_L vectors were induced at 37°C in a shaking water bath for 4, 9, or 20 hours, and the preparations were sonicated and heat treated at 75°C in the presence of 0.2 M ammonium
15 sulfate. Finally, the extracts were assayed for polymerase activity and 5' to 3' exonuclease activity.

The 5' to 3' exonuclease activity was quantified utilizing the 5' to 3' exonuclease assay described above. Specifically, the synthetic 3' phosphorylated
20 oligonucleotide probe (phosphorylated to preclude polymerase extension) BW33 (GATCGCTGCGCGTAACCACCA-CACCCGCCGCGCp) (SEQ ID NO:13) (100 pmol) was ³²P-labeled at the 5' end with gamma-[³²P] ATP (3000 Ci/mmol) and T4 polynucleotide kinase. The reaction
25 mixture was extracted with phenol:chloroform:isoamyl alcohol, followed by ethanol precipitation. The ³²P-labeled oligonucleotide probe was redissolved in 100 μ l of TE buffer, and unincorporated ATP was removed by gel filtration chromatography on a Sephadex G-50
30 spin column. Five pmol of ³²P-labeled BW33 probe, was annealed to 5 pmol of single-strand M13mp10w DNA, in the presence of 5 pmol of the synthetic oligonucleotide primer BW37 (GCGCTAGGGCGCTGGCAAGTGTAGCGGTCA) (SEQ ID NO:14) in a 100 μ l reaction containing 10 mM Tris-HCl
35 (pH 8.3), 50 mM KCl, and 3 mM MgCl₂. The annealing mixture was heated to 95°C for 5 minutes, cooled to

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70°C over 10 minutes, incubated at 70°C for an additional 10 minutes, and then cooled to 25°C over a 30 minute period in a Perkin-Elmer Cetus DNA thermal cycler. Exonuclease reactions containing 10 µl of the 5 annealing mixture were pre-incubated at 70°C for 1 minute. The thermostable DNA polymerase preparations of the invention (approximately 0.3 U of enzyme activity) were added in a 2.5 µl volume to the pre-incubation reaction, and the reaction mixture was 10 incubated at 70°C. Aliquots (5 µl) were removed after 1 minute and 5 minutes, and stopped by the addition of 1 µl of 60 mM EDTA. The reaction products were analyzed by homochromatography and exonuclease activity was quantified following autoradiography. 15 Chromatography was carried out in a homochromatography mix containing 2% partially hydrolyzed yeast RNA in 7M urea on Polygram CEL 300 DEAE cellulose thin layer chromatography plates. The presence of 5' to 3' exonuclease activity resulted in the generation of 20 small ³²P-labeled oligomers, which migrated up the TLC plate, and were easily differentiated on the autoradiogram from undegraded probe, which remained at the origin.

The clone 3-2 had an expected level of polymerase 25 activity but barely detectable 5' to 3' exonuclease activity. This represented a greater than 1000-fold reduction in 5' to 3' exonuclease activity from that present in native Taq DNA polymerase.

This clone was then sequenced and it was found that 30 G (137) was mutated to an A in the DNA sequence. This mutation results in a Gly (46) to Asp mutation in the amino acid sequence of the Taq DNA polymerase, thus yielding a thermostable DNA polymerase of the present invention with significantly attenuated 5' to 3' 35 exonuclease activity.

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The recovered protein was purified according to the Tag DNA polymerase protocol which is taught in Serial No. 523,394 filed May 15, 1990, incorporated herein by reference.

5

Example 2

Construction of Met 289 (Δ 289) 544 Amino Acid Form of Tag Polymerase

10

As indicated in Example 9 of U.S. Serial No. 523,394, filed May 15, 1990, during a purification of native Tag polymerase an altered form of Tag polymerase was obtained that catalyzed the template dependent
15 incorporation of dNTP at 70°C. This altered form of Tag polymerase was immunologically related to the approximate 90 kd form of purified native Tag polymerase but was of lower molecular weight. Based on mobility, relative to BSA and ovalbumin following
20 SDS-PAGE electrophoresis, the apparent molecular weight of this form is approximately 61 kd. This altered form of the enzyme is not present in carefully prepared crude extracts of Thermus aquaticus cells as determined by SDS-PAGE Western blot analysis or in situ DNA
25 polymerase activity determination (Spanos, A., and Hubscher, U. (1983) Meth. Enz. 91:263-277) following SDS-PAGE gel electrophoresis. This form appears to be a proteolytic artifact that may arise during sample handling. This lower molecular weight form was
30 purified to homogeneity and subjected to N-terminal sequence determination on an ABI automated gas phase sequencer. Comparison of the obtained N-terminal sequence with the predicted amino acid sequence of the Tag polymerase gene (SEQ ID NO:1) indicates this
35 shorter form arose as a result of proteolytic cleavage between Glu(289) and Ser(290).

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To obtain a further truncated form of a Taq polymerase gene that would direct the synthesis of a 544 amino acid primary translation production plasmids pFC54.t, pSYC1578 and the complementary synthetic oligonucleotides DG29 (5'-AGCTTATGTCTCCAAAAGCT) (SEQ ID NO:23) and DG30 (5'-AGCTTTTGGAGACATA) (SEQ ID NO:24) were used. Plasmid pFC54.t was digested to completion with HindIII and BamHI. Plasmid pSYC1578 was digested with BstXI (at nucleotides 872 to 883 of SEQ ID NO:1) and treated with E. coli DNA polymerase I Klenow fragment in the presence of all 4 dNTPs to remove the 4 nucleotide 3' cohesive end and generate a CTG-terminated duplex blunt end encoding Leu294 in the Taq polymerase sequence (see Taq polymerase SEQ ID NO:1 nucleotides 880-882). The DNA sample was digested to completion with BglII and the approximate 1.6 kb BstXI (repaired)/BglII Taq DNA fragment was purified by agarose gel electrophoresis and electroelution. The pFC54.t plasmid digest (0.1 pmole) was ligated with the Taq polymerase gene fragment (0.3 pmole) and annealed nonphosphorylated DG29/DG30 duplex adaptor (0.5 pmole) under sticky ligase conditions at 30 µg/ml, 15°C overnight. The DNA was diluted to approximately 10 microgram per ml and ligation continued under blunt end conditions. The ligated DNA sample was digested with XbaI to linearize (inactivate) any IL-2 mutein-encoding ligation products. 80 nanograms of the ligated and digested DNA was used to transform E. coli K12 strain DG116 to ampicillin resistance. Amp^R candidates were screened for the presence of an approximate 7.17 kb plasmid which yielded the expected digestion products with EcoRI (4,781 bp + 2,386 bp), PstI (4,138 bp + 3,029 bp), ApaI (7,167 bp) and HindIII/PstI (3,400 bp + 3,029 bp + 738 bp). E. coli colonies harboring candidate plasmids were screened by single colony immunoblot for the temperature-inducible synthesis of

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an approximate 61 kd Taq polymerase related polypeptide. In addition, candidate plasmids were subjected to DNA sequence determination at the 5' λP_L promoter:Taq DNA junction and the 3' Taq DNA:BT cry PRE 5 junction. One of the plasmids encoding the intended DNA sequence and directing the synthesis of a temperature-inducible 61 kd Taq polymerase related polypeptide was designated pLSG68.

Expression of 61 kDa Taq Pol I. Cultures 10 containing pLSG8 were grown as taught in Serial No. 523,364 and described in Example 3 below. The 61 kDa Taq Pol I appears not to be degraded upon heat-induction at 41°C. After 21 hours at 41°C, a heat-treated crude extract from a culture harboring 15 pLSG8 had 12,310 units of heat-stable DNA polymerase activity per mg crude extract protein, a 24-fold increase over an uninduced culture. A heat-treated extract from a 21 hour 37°C-induced pLSG8 culture had 9,503 units of activity per mg crude extract protein. 20 A nine-fold increase in accumulated levels of Taq Pol I was observed between a 5 hour and 21 hour induction at 37°C and a nearly four-fold increase between a 5 hour and 21 hour induction at 41°C. The same total protein and heat-treated extracts were analyzed by SDS-PAGE. 25 20 μ g crude extract protein or heat-treated crude extract from 20 μ g crude extract protein were applied to each lane of the gel. The major bands readily apparent in both the 17°C and 41°C, 21 hour-induced total protein lanes are equally intense as their 30 heat-treated counterparts. Heat-treated crude extracts from 20 μ g of total protein from 37°C and 41°C, 21 hour samples contain 186 units and 243 units of thermostable DNA polymerase activity, respectively. To determine the usefulness of 61 kDa Taq DNA polymerase in PCR, PCR 35 assays were performed using heat-treated crude extracts from induced cultures of pLSG8. Heat-treated crude

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extract from induced cultures of pLSG5 were used as the source of full-length Tag Pol I in PCR. PCR product was observed in reactions utilizing 4 units and 2 units of truncated enzyme. There was more product in those 5 PCRs than in any of the full-length enzyme reactions. In addition, no non-specific higher molecular weight products were visible.

Purification of 61 kDa Tag Pol I. Purification of 61 kDa Tag Pol I from induced pLSG8/DG116 cells 10 proceeded as the purification of full-length Tag Pol I as in Example 12 of U.S. Serial No. 523,394, filed May 15, 1990 with some modifications.

Induced pLSG8/DG116 cells (15.6 g) were homogenized and lysed as described in U.S. Serial No. 523,394, 15 filed May 15, 1990 and in Example 3 below. Fraction I contained 1.87 g protein and 1.047×10^6 units of activity. Fraction II, obtained as a 0.2 M ammonium sulfate supernant contained 1.84 g protein and 1.28×10^6 units of activity in 74 ml.

20 Following heat treatment, Polymix P (pH 7.5) was added slowly to 0.7%. Following centrifugation, the supernant, Fraction III contained 155 mg protein and 1.48×10^6 units of activity.

Fraction III was loaded onto a 1.15 x 3.1 cm (3.2 25 ml) phenyl sepharose column at 10 ml/cm²/hour. All of the applied activity was retained on the column. The column was washed with 15 ml of the equilibration buffer and then 5 ml (1.5 column volumes) of 0.1M KCl in TE. The polymerase activity was eluted with 2 M 30 urea in TE containing 20% ethylene glycol. Fractions (0.5 ml each) with polymerase activity were pooled (8.5 ml) and dialyzed into heparin sepharose buffer containing 0.1 M KCl. The dialyzed material, Fraction IV (12.5 ml), contained 5.63 mg of protein and $1.29 \times$ 35 10^6 units of activity.

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Fraction IV was loaded onto a 1.0 ml bed volume heparin sepharose column equilibrated as above. The column was washed with 6 ml of the same buffer (A_{280} to baseline) and eluted with a 15 ml linear 0.1-0.5 M KCl gradient in the same buffer. Fractions (0.15 ml) eluting between 0.16 and 0.27 M KCl were analyzed by SDS-PAGE. A minor (<1%) contaminating approximately 47 kDa protein copurified with 61 kDa Tag Pol I. Fractions eluting between 0.165 and 0.255 M KCl were 10 pooled (2.5 ml) and diafiltered on a Centricon 30 membrane into 2.5X storage buffer. Fraction V contained 2.8 mg of protein and 1.033×10^6 units of 61 kDa Tag Pol I.

PCR Using Purified 61 kDa Tag Pol I. PCR reactions 15 (50 μ l) containing 0.5 ng lambda DNA, 10 pmol each of two lambda-specific primers, 200 μ M each dNTPs, 10 mM Tris-Cl, pH 8.3, 3 mM $MgCl_2$, 10 mM KCl and 3.5 units of 61 kDa Tag Pol I were performed. As a comparison, PCR reactions were performed with 1.25 units of full-length 20 Tag Pol I, as above, with the substitution of 2 mM $MgCl_2$ and 50 mM KCl. Thermocycling conditions were 1 minute at 95°C and 1 minute at 60°C for 23 cycles, with a final 5 minute extension at 75°C. The amount of DNA per reaction was quantitated by the Hoechst fluorescent 25 dye assay. 1.11 μ g of product was obtained with 61 kDa Tag Pol I (2.2×10^5 -fold amplification), as compared with 0.70 μ g of DNA with full-length Tag Pol I (1.4×10^5 -fold amplification).

Thermostability of 61 kDa Tag Pol I. Steady state 30 thermal inactivation of recombinant 94 kDa Tag Pol I and 61 kDa Tag Pol I was performed 97.5°C under buffer conditions mimicking PCR. 94 kDa Tag Pol I has an apparent half-life of approximately 9 minute at 97.5°C, whereas the half-life of 61 kDa Tag Pol I was

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approximately 21 minutes. The thermal inactivation of 61 kDa Taq Pol I was unaffected by KCl concentration over a range from 0 to 50 mM.

Yet another truncated Taq polymerase gene contained 5 within the ~2.68 kb HindIII-Asp718 fragment of plasmid pFC85 can be expressed using, for example, plasmid pP_LN_{RBS}ATG, by operably linking the amino-terminal HindIII restriction site encoding the Taq pol gene to an ATG initiation codon. The product of this fusion 10 upon expression will yield an ~70,000-72,000 dalton truncated polymerase.

This specific construction can be made by digesting plasmid pFC85 with HindIII and treating with Klenow fragment in the presence of dATP and dGTP. The 15 resulting fragment is treated further with S1 nuclease to remove any single-stranded extensions and the resulting DNA digested with Asp718 and treated with Klenow fragment in the presence of all four dNTPs. The recovered fragment can be ligated using T4 DNA ligase 20 to dephosphorylated plasmid pP_LN_{RBS}ATG, which had been digested with SacI and treated with Klenow fragment in the presence of dGTP to construct an ATG blunt end. This ligation mixture can then be used to transform E. coli DG116 and the transformants screened for 25 production of Taq polymerase. Expression can be confirmed by Western immunoblot analysis and activity analysis.

Example 3

30

Construction, Expression and Purification of a Truncated 5' to 3' Exonuclease Deficient Tma Polymerase (MET284)

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To express a 5' to 3' exonuclease deficient Tma DNA polymerase lacking amino acids 1-283 of native Tma DNA polymerase the following steps were performed.

Plasmid pTma12-1 was digested with BspHI 5 (nucleotide position 848) and HindIII (nucleotide position 2629). A 1781 base pair fragment was isolated by agarose gel purification. To separate the agarose from the DNA, a gel slice containing the desired fragment was frozen at -20°C in a Costar spinex filter 10 unit. After thawing at room temperature, the unit was spun in a microfuge. The filtrate containing the DNA was concentrated in a Speed Vac concentrator, and the DNA was precipitated with ethanol.

The isolated fragment was cloned into plasmid 15 pTma12-1 digested with NcoI and HindIII. Because NcoI digestion leaves the same cohesive end sequence as digestion with BspHI, the 1781 base pair fragment has the same cohesive ends as the full length fragment excised from plasmid pTma12-1 by digestion with NcoI 20 and HindIII. The ligation of the isolated fragment with the digested plasmid results in a fragment switch and was used to create a plasmid designated pTma14.

Plasmid pTma15 was similarly constructed by cloning the same isolated fragment into pTma13. As with 25 pTma14, pTma15 drives expression of a polymerase that lacks amino acids 1 through 283 of native Tma DNA polymerase; translation initiates at the methionine codon at position 284 of the native coding sequence.

Both the pTma14 and pTma15 expression plasmids 30 expressed at a high level a biologically active thermostable DNA polymerase devoid of 5' to 3' exonuclease activity of molecular weight of about 70 kDa; plasmid pTma15 expressed polymerase at a higher level than did pTma14. Based on similarities with E. 35 coli Pol I Klenow fragment, such as conservation of amino acid sequence motifs in all three domains that

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are critical for 3' to 5' exonuclease activity, distance from the amino terminus to the first domain critical for exonuclease activity, and length of the expressed protein, the shortened form (MET284) of Tma DNA polymerase exhibits 3' to 5' exonuclease or proof-reading activity but lacks 5' to 3' exonuclease activity. Initial SDS activity gel assays and solution assays for 3' to 5' exonuclease activity suggest attenuation in the level of proof-reading activity of the polymerase expressed by E. coli host cells harboring plasmid pTma15.

MET284 Tma DNA polymerase was purified from E. coli strain DG116 containing plasmid pTma15. The seed flask for a 10 L fermentation contained tryptone (20 g/l), yeast extract (10 g/l), NaCl (10 g/l), glucose (10 g/l), ampicillin (50 mg/l), and thiamine (10 mg/l). The seed flask was inoculated with a colony from an agar plate (a frozen glycerol culture can be used). The seed flask was grown at 30°C to between 0.5 to 2.0 O.D. (A₆₈₀). The volume of seed culture inoculated into the fermentor is calculated such that the bacterial concentration is 0.5 mg dry weight/liter. The 10 liter growth medium contained 25 mM KH₂PO₄, 10 mM (NH₄)₂SO₄, 4 mM sodium citrate, 0.4 mM FeCl₃, 0.04 mM ZnCl₂, 0.03 mM CoCl₂, 0.03 mM CuCl₂, and 0.03 mM H₃BO₃. The following sterile components were added: 4 mM MgSO₄, 20 g/l glucose, 20 mg/l thiamine, and 50 mg/l ampicillin. The pH was adjusted to 6.8 with NaOH and controlled during the fermentation by added NH₄OH. Glucose was continually added by coupling to NH₄OH addition. Foaming was controlled by the addition of propylene glycol as necessary, as an antifoaming agent. Dissolved oxygen concentration was maintained at 40%.

The fermentor was inoculated as described above, and the culture was grown at 30°C to a cell density of 0.5 to 1.0 X 10¹⁰ cells/ml (optical density [A₆₈₀] of

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15). The growth temperature was shifted to 38°C to induce the synthesis of MET284 Tma DNA polymerase. The temperature shift increases the copy number of the pTma15 plasmid and simultaneously derepresses the 5 lambda P_L promoter controlling transcription of the modified Tma DNA polymerase gene through inactivation of the temperature-sensitive cI repressor encoded by the defective prophage lysogen in the host.

The cells were grown for 6 hours to an optical 10 density of 37 (A₆₈₀) and harvested by centrifugation. The cell mass (ca. 95 g/l) was resuspended in an equivalent volume of buffer containing 50 mM Tris-Cl, pH 7.6, 20 mM EDTA and 20% (w/v) glycerol. The suspension was slowly dripped into liquid nitrogen to 15 freeze the suspension as "beads" or small pellets. The frozen cells were stored at -70°C.

To 200 g of frozen beads (containing 100 g wet weight cell) were added 100 ml of 1X TE (50 mM Tris-Cl, pH 7.5, 10 mM EDTA) and DTT to 0.3 mM, PMSF to 2.4 mM, 20 leupeptin to 1 µg/ml and TLCK (a protease inhibitor) to 0.2 mM. The sample was thawed on ice and uniformly resuspended in a blender at low speed. The cell suspension was lysed in an Aminco french pressure cell at 20,000 psi. To reduce viscosity, the lysed cell 25 sample was sonicated 4 times for 3 min. each at 50% duty cycle and 70% output. The sonicate was adjusted to 550 ml with 1X TE containing 1 mM DTT, 2.4 mM PMSF, 1 µg/ml leupeptin and 0.2 mM TLCK (Fraction I). After addition of ammonium sulfate to 0.3 M, the crude lysate 30 was rapidly brought to 75°C in a boiling water bath and transferred to a 75°C water bath for 15 min. to denature and inactivate E. coli host proteins. The heat-treated sample was chilled rapidly to 0°C and incubated on ice for 20 min. Precipitated proteins and

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cell membranes were removed by centrifugation at 20,000 X G for 30 min. at 5°C and the supernatant (Fraction II) saved.

The heat-treated supernatant (Fraction II) was treated with polyethyleneimine (PEI) to remove most of the DNA and RNA. Polymix P (34.96 ml of 10% [w/v], pH 7.5) was slowly added to 437 ml of Fraction II at 0°C while stirring rapidly. After 30 min. at 0°C, the sample was centrifuged at 20,000 X G for 30 min. The supernatant (Fraction III) was applied at 80 ml/hr to a 100 ml phenylsepharose column (3.2 x 12.5 cm) that had been equilibrated in 50 mM Tris-Cl, pH 7.5, 0.3 M ammonium sulfate, 10 mM EDTA, and 1 mM DTT. The column was washed with about 200 ml of the same buffer (A₂₈₀ to baseline) and then with 150 ml of 50 mM Tris-Cl, pH 7.5, 100 mM KCl, 10 mM EDTA and 1 mM DTT. The MET284 Tma DNA polymerase was then eluted from the column with buffer containing 50 mM Tris-Cl, pH 7.5, 2 M urea, 20% (w/v) ethylene glycol, 10 mM EDTA, and 1 mM DTT, and 20 fractions containing DNA polymerase activity were pooled (Fraction IV).

Fraction IV is adjusted to a conductivity equivalent to 50 mM KCl in 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 1 mM DTT. The sample was applied (at 9 ml/hr) to a 15 ml heparin-sepharose column that had been equilibrated in the same buffer. The column was washed with the same buffer at ca. 14 ml/hr (3.5 column volumes) and eluted with a 150 ml 0.05 to 0.5 M KCl gradient in the same buffer. The DNA polymerase activity eluted between 0.11-0.22 M KCl. Fractions containing the pTma15 encoded modified Tma DNA polymerase are pooled, concentrated, and diafiltered against 2.5X storage buffer (50 mM Tris-Cl, pH 8.0, 250 mM KCl, 0.25 mM EDTA, 2.5 mM DTT, and 0.5% Tween 20), subsequently mixed with 1.5 volumes of sterile 80% (w/v) glycerol, and stored at -20°C. Optionally, the

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heparin sepharose-eluted DNA polymerase or the phenyl sepharose-eluted DNA polymerase can be dialyzed or adjusted to a conductivity equivalent to 50 mM KCl in 50 mM Tris-Cl, pH 7.5, 1 mM DTT, 1 mM EDTA, and 0.2% Tween 20 and applied (1 mg protein/ml resin) to an affigel blue column that has been equilibrated in the same buffer. The column is washed with three to five column volumes of the same buffer and eluted with a 10 column volume KCl gradient (0.05 to 0.8 M) in the same 10 buffer. Fractions containing DNA polymerase activity (eluting between 0.25 and 0.4 M KCl) are pooled, concentrated, diafiltered, and stored as above.

The relative thermoresistance of various DNA polymerases has been compared. At 97.5°C the half-life 15 of native Tma DNA polymerase is more than twice the half-life of either native or recombinant Taq DNA (i.e., AmpliTaq) DNA polymerase. Surprisingly, the half-life at 97.5°C of MET284 Tma DNA polymerase is 2.5 to 3 times longer than the half-life of native Tma DNA 20 polymerase.

PCR tubes containing 10 mM Tris-Cl, pH 8.3, and 1.5 mM MgCl₂ (for Taq or native Tma DNA polymerase) or 3 mM MgCl₂ (for MET284 Tma DNA polymerase), 50 mM KCl (for Taq, native Tma and MET284 Tma DNA polymerases) or no 25 KCl (for MET284 Tma DNA polymerase), 0.5 µM each of primers PCR01 and PCR02, 1 ng of lambda template DNA, 200 µM of each dNTP except dCTP, and 4 units of each enzyme were incubated at 97.5°C in a large water bath for times ranging from 0 to 60 min. Samples were 30 withdrawn with time, stored at 0°C, and 5 µl assayed at 75°C for 10 min. in a standard activity assay for residual activity.

Taq DNA polymerase had a half-life of about 10 min. at 97.5°C, while native Tma DNA polymerase had a 35 half-life of about 21 to 22 min. at 97.5°C. Surprisingly, the MET284 form of Tma DNA polymerase had

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a significantly longer half-life (50 to 55 min.) than either Tag or native Tma DNA polymerase. The improved thermoresistance of MET284 Tma DNA polymerase will find applications in PCR, particularly where G+C-rich targets are difficult to amplify because the strand-separation temperature required for complete denaturation of target and PCR product sequences leads to enzyme inactivation.

PCR tubes containing 50 μ l of 10 mM Tris-Cl, pH 8.3, 3 mM $MgCl_2$, 200 μ M of each dNTP, 0.5 ng bacteriophage lambda DNA, 0.5 μ M of primer PCR01, 4 units of MET284 Tma DNA polymerase, and 0.5 μ M of primer PCR02 or PL10 were cycled for 25 cycles using T_{den} of 96°C for 1 min. and $T_{anneal-extend}$ of 60°C for 2 min. Lambda DNA template, deoxynucleotide stock solutions, and primers PCR01 and PCR02 were part of the PEFI GeneAmp kit. Primer PL10 has the sequence: 5'-GGCGTACCTTTGTCTCACGGGCAAC-3' (SEQ ID NO:25) and is complementary to bacteriophage lambda nucleotides 8106-8130.

The primers PCR01 and PCR02 amplify a 500 bp product from lambda. The primer pair PCR01 and PL10 amplify a 1 kb product from lambda. After amplification with the respective primer sets, 5 μ l aliquots were subjected to agarose gel electrophoresis and the specific intended product bands visualized with ethidium bromide staining. Abundant levels of product were generated with both primer sets, showing that MET284 Tma DNA polymerase successfully amplified the intended target sequence.

Example 4Expression of Truncated Tma DNA Polymerase

5 To express a 5' to 3' exonuclease deficient form of
 Tma DNA polymerase which initiates translation at MET
 140 the coding region corresponding to amino acids 1
 through 139 was deleted from the expression vector.
 The protocol for constructing such a deletion is
10 similar to the construction described in Examples 2
 and 3: a shortened gene fragment is excised and then
 reinserted into a vector from which a full length
 fragment has been excised. However, the shortened
 fragment can be obtained as a PCR amplification product
15 rather than purified from a restriction digest. This
 methodology allows a new upstream restriction site (or
 other sequences) to be incorporated where useful.

 To delete the region up to the methionine codon at
 position 140, an SphI site was introduced into pTma12-1
20 and pTma13 using PCR. A forward primer corresponding
 to nucleotides 409-436 of Tma DNA polymerase SEQ ID
 NO:3 (FL63) was designed to introduce an SphI site just
 upstream of the methionine codon at position 140. The
 reverse primer corresponding to the complement of
25 nucleotides 608-634 of Tma DNA polymerase SEQ ID NO:3
 (FL69) was chosen to include an XbaI site at position
 621. Plasmid pTma12-1 linearized with SmaI was used as
 the PCR template, yielding an approximate 225 bp PCR
 product.

30 Before digestion, the PCR product was treated with
 50 µg/ml of Proteinase K in PCR reaction mix plus 0.5%
 SDS and 5 mM EDTA. After incubating for 30 minutes at
 37°C, the Proteinase K was heat inactivated at 68°C for
 10 minutes. This procedure eliminated any Tag
35 polymerase bound to the product that could inhibit

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subsequent restriction digests. The buffer was changed to a TE buffer, and the excess PCR primers were removed with a Centricon 100 microconcentrator.

The amplified fragment was digested with SphI, then
 5 treated with Klenow to create a blunt end at the
SphI-cleaved end, and finally digested with XbaI. The
 resulting fragment was ligated with plasmid pTma13
 (pTma12-1 would have been suitable) that had been
 digested with NcoI, repaired with Klenow, and then
 10 digested with XbaI. The ligation yielded an in-frame
 coding sequence with the region following the NcoI site
 (at the first methionine codon of the coding sequence)
 and the introduced SphI site (upstream of the
 methionine codon at position 140) deleted. The
 15 resulting expression vector was designated pTma16.

The primers used in this example are given below
 and in the Sequence Listing section.

<u>Primer</u>	<u>SEQ ID NO:</u>	<u>Sequence</u>
20		
FL63	SEQ ID NO:26	5'GATAAAGGCATGCTTCAGCTTGTGAACG
FL69	SEQ ID NO:27	5'TGTACTTCTCTAGAAGCTGAACAGCAG

Example 5

25

Elimination of Undesired RBS in MET140 Expression Vectors

Reduced expression of the MET140 form of Tma DNA
 30 polymerase can be achieved by eliminating the ribosome
 binding site (RBS) upstream of the methionine codon at
 position 140. The RBS was be eliminated via
 oligonucleotide site-directed mutagenesis without
 changing the amino acid sequence. Taking advantage of
 35 the redundancy of the genetic code, one can make
 changes in the third position of codons to alter the

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nucleic acid sequence, thereby eliminating the RBS, without changing the amino acid sequence of the encoded protein.

A mutagenic primer (FL64) containing the modified 5 sequence was synthesized and phosphorylated. Single-stranded pTma09 (a full length clone having an NcoI site) was prepared by coinfecting with the helper phage R408, commercially available from Stratagene. A "gapped duplex" of single stranded pTma09 and the large 10 fragment from the PvuII digestion of pBS13+ was created by mixing the two plasmids, heating to boiling for 2 minutes, and cooling to 65°C for 5 minutes. The phosphorylated primer was then annealed with the "gapped duplex" by mixing, heating to 80°C for 2 15 minutes, and then cooling slowly to room temperature. The remaining gaps were filled by extension with Klenow and the fragments ligated with T4 DNA ligase, both reactions taking place in 200 µM of each dNTP and 40 µM ATP in standard salts at 37°C for 30 minutes.

20 The resulting circular fragment was transformed into DG101 host cells by plate transformations on nitrocellulose filters. Duplicate filters were made and the presence of the correct plasmid was detected by probing with a $\gamma^{32}\text{P}$ -phosphorylated probe (FL65). The 25 vector that resulted was designated pTma19.

The RBS minus portion from pTma19 was cloned into pTma12-1 via an NcoI/XbaI fragment switch. Plasmid pTma19 was digested with NcoI and XbaI, and the 620 bp fragment was purified by gel electrophoresis, as in 30 Example 3, above. Plasmid pTma12-1 was digested with NcoI, XbaI, and XcmI. The XcmI cleavage inactivates the RBS+ fragment for the subsequent ligation step, which is done under conditions suitable for ligating "sticky" ends (dilute ligase and 40 µM ATP). Finally, 35 the ligation product is transformed into DG116 host cells for expression and designated pTma19-RBS.

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The oligonucleotide sequences used in this example are listed below and in the Sequence Listing section.

<u>Oligo</u>	<u>SEQ ID NO:</u>	<u>Sequence</u>
5		
FL64	SEQ ID NO:28	5'CTGAAGCATGTCTTTGTCACCGGT-
		TACTATGAATAT
FL65	SEQ ID NO:29	5'TAGTAACCGGTGACAAAG

10

Example 6

Expression of Truncated Tma DNA Polymerases
MET-ASP21 and MET-GLU74

15 To effect translation initiation at the aspartic acid codon at position 21 of the Tma DNA polymerase gene coding sequence, a methionine codon is introduced before the codon, and the region from the initial NcoI site to this introduced methionine codon is deleted. Similar to

20 Example 4, the deletion process involved PCR with the same downstream primer described above (FL69) and an upstream primer (FL66) designed to incorporate an NcoI site and a methionine codon to yield a 570 base pair product.

25 The amplified product was concentrated with a Centricon-100 microconcentrator to eliminate excess primers and buffer. The product was concentrated in a Speed Vac concentrator and then resuspended in the digestion mix. The amplified product was digested with

30 NcoI and XbaI. Likewise, pTma12-1, pTma13, or pTma19-RBS was digested with the same two restriction enzymes, and the digested, amplified fragment is ligated with the digested expression vector. The resulting construct has a deletion from the NcoI site upstream of

35 the start codon of the native Tma coding sequence to the

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new methionine codon introduced upstream of the aspartic acid codon at position 21 of the native Tma coding sequence.

Similarly, a deletion mutant was created such that translation initiation begins at Glu74, the glutamic acid codon at position 74 of the native Tma coding sequence. An upstream primer (FL67) is designed to introduce a methionine codon and an NcoI site before Glu74. The downstream primer and cloning protocol used are as described above for the MET-ASP21 construct.

The upstream primer sequences used in this example are listed below and in the Sequence Listing section.

<u>Oligo</u>	<u>SEQ ID NO:</u>	<u>Sequence</u>
15		
FL66	SEQ ID NO:30	5'CTATGCCATGGATAGATCGCTT-TCTACTTCC
FL67	SEQ ID NO:31	5'CAAGCCCATGGAACTTACAAG-GCTCAAAGA
20		

Example 7

Expression of Truncated Taf Polymerase

Mutein forms of the Taf polymerase lacking 5' to 3' exonuclease activity were constructed by introducing deletions in the 5' end of the Taf polymerase gene. Both 279 and 417 base pair deletions were created using the following protocol; an expression plasmid was digested with restriction enzymes to excise the desired fragment, the fragment ends were repaired with Klenow and all four dNTP/s, to produce blunt ends, and the products were ligated to produce a new circular plasmid with the desired deletion. To express a 93 kilodalton, 5' to 3' exonuclease-deficient form of Taf polymerase, a 279 bp deletion comprising amino acids 2-93 was

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generated. To express an 88 kilodalton, 5' to 3' exonuclease-deficient form of Taf polymerase, 417 bp deletion comprising amino acids 2-139 was generated.

To create a plasmid with codons 2-93 deleted, 5 pTaf03 was digested with NcoI and NdeI and the ends were repaired by Klenow treatment. The digested and repaired plasmid was diluted to 5 µg/ml and ligated under blunt end conditions. The dilute plasmid concentration favors intramolecular ligations. The 10 ligated plasmid was transformed into DG116. Mini-screen DNA preparations were subjected to restriction analysis and correct plasmids were confirmed by DNA sequence analysis. The resulting expression vector created by deleting a segment from 15 pTaf03 was designated pTaf09. A similar vector created from pTaf05 was designated pTaf10.

Expression vectors also were created with codons 2-139 deleted. The same protocol was used with the exception that the initial restriction digestion was 20 performed with NcoI and BglII. The expression vector created from pTaf03 was designated pTaf11 and the expression vector created from pTaf05 was designated pTaf12.

25

Example 8

Derivation and Expression of 5' to 3'
Exonuclease-Deficient, Thermostable DNA
Polymerase of Thermus species, Z05
30 Comprising Amino Acids 292 Through 834

To obtain a DNA fragment encoding a 5' to 3' exonuclease-deficient thermostable DNA polymerase from Thermus species Z05, a portion of the DNA polymerase 35 gene comprising amino acids 292 through 834 is selectively amplified in a PCR with forward primer TZA292 and reverse primer TZR01 as follows:

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50 pmoles TZA292
50 pmoles TZR01
10 ng Thermus sp. Z05 genomic DNA
2.5 units AmpliTaq DNA polymerase
5 50 μ M each dATP, dGTP, dCTP, dTTP

in an 80 μ l solution containing 10 mM Tris-HCl pH 8.3,
50 mM KCl and overlaid with 100 μ l of mineral oil. The
reaction was initiated by addition of 20 μ l containing
10 7.5 mM MgCl₂ after the tubes had been placed in an 80°C
preheated cyclor.

The genomic DNA was digested to completion with
restriction endonuclease Asp718, denatured at 98°C for
5 minutes and cooled rapidly to 0°C. The sample was
15 cycled in a Perkin-Elmer Cetus Thermal Cyclor according
to the following profile:

STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 55°C and hold for 30 seconds.
20 RAMP to 72°C over 30 seconds and hold for 1 minute.
REPEAT profile for 3 cycles.

STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 65°C and hold for 2 minutes.
25 REPEAT profile for 25 cycles.
After last cycle HOLD for 5 minutes.

The intended 1.65 kb PCR product is purified by
agarose gel electrophoresis, and recovered following
30 phenol-chloroform extraction and ethanol precipitation.
The purified product is digested with restriction
endonucleases NdeI and BglII and ligated with
NdeI/BamHI-digested and dephosphorylated plasmid vector
pDG164 (U.S. Serial No. 455,967, filed December 22,
35 1989, Example 6B incorporated herein by reference).
Ampicillin-resistant transformants of E. coli strain

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DG116 are selected at 30°C and screened for the desired recombinant plasmid. Plasmid pZ05A292 encodes a 544 amino acid, 5' to 3' exonuclease-deficient Thermus sp. Z05 thermostable DNA polymerase analogous to the pLSG8 encoded protein of Example 2. The DNA polymerase activity is purified as in Example 2. The purified protein is deficient in 5' to 3' exonuclease activity, is more thermoresistant than the corresponding native enzyme and is particularly useful in PCR of G+C-rich templates.

<u>Primer</u>	<u>SEQ ID NO:</u>	<u>SEQUENCE</u>
TZA292	SEQ ID NO:32	GTCGGCATATGGCTCCTGCTCCTCTTGAGGA- GGCCCCCTGGCCCCCGCC
TZR01	SEQ ID NO:33	GACGCAGATCTCAGCCCTTGGCGGAAAGCCA- GTCCTC

20

Example 9

Derivation and Expression of 5' to 3'
Exonuclease-Deficient, Thermostable DNA
Polymerase of Thermus species SPS17
Comprising Amino Acids 288 Through 830

25

To obtain a DNA fragment encoding 5' to 3' exonuclease-deficient thermostable DNA polymerase from Thermus species SPS17, a portion of the DNA polymerase gene comprising amino acids 288 through 830 is selectively amplified in a PCR with forward primer TSA288 and reverse primer TSR01 as follows:

30

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50 pmoles TSA288
50 pmoles TSR01
10 ng Thermus sp. SPS17 genomic DNA
2.5 units AmpliTaq DNA polymerase
5 50 µM each dATP, dGTP, dCTP, dTTP

in an 80 µl solution containing 10 mM Tris-HCl pH 8.3,
50 mM KCl and overlaid with 100 µl of mineral oil. The
reaction was initiated by addition of 20 µl containing
10 7.5 mM MgCl₂ after the tubes had been placed in an 80°C
preheated cyclor.

The genomic DNA was denatured at 98°C for 5 minutes
and cooled rapidly to 0°C. The sample was cycled in a
15 Perkin-Elmer Cetus Thermal Cyclor according to the
following profile:

STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 55°C and hold for 30 seconds.
20 RAMP to 72°C over 30 seconds and hold for 1 minute.
REPEAT profile for 3 cycles.

STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 65°C and hold for 2 minutes.
25 REPEAT profile for 25 cycles.
After last cycle HOLD for 5 minutes.

The intended 1.65 kb PCR product is purified by
agarose gel electrophoresis, and recovered following
30 phenol-chloroform extraction and ethanol precipitation.
The purified product is digested with restriction
endonucleases NdeI and BglII and ligated with
NdeI/BamHI-digested and dephosphorylated plasmid vector
pDG164 (U.S. Serial No. 455,967, filed December 12,
35 1989, Example 6B). Ampicillin- resistant transformants
of E. coli strain DG116 are selected at 30°C and

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screened for the desired recombinant plasmid. Plasmid pSPSA288 encodes a 544 amino acid, 5' to 3' exonuclease-deficient Thermus sp. SPS17 thermostable DNA polymerase analogous to the pLSG8 encoded protein of Example 2. The DNA polymerase activity is purified as in Example 2. The purified protein is deficient in 5' to 3' exonuclease activity, is more thermoresistant than the corresponding native enzyme and is particularly useful in PCR of G+C-rich templates.

10

<u>Primer</u>	<u>SEQ ID NO:</u>	<u>SEQUENCE</u>
TSA288	SEQ ID NO:34	GTCGGCATATGGCTCCTAAAGAAGCTGAGGA- GGCCCCCTGGCCCCCGCC
TSR01	SEQ ID NO:35	GACGCAGATCTCAGGCCTTGGCGGAAAGCCA- GTCCTC

15

Example 10

20

Derivation and Expression of 5' to 3'
Exonuclease-Deficient, Thermostable DNA
Polymerase of Thermus Thermophilus
Comprising Amino Acids 292 Through 834

25

To obtain a DNA fragment encoding a 5' to 3' exonuclease-deficient thermostable DNA polymerase from Thermus thermophilus, a portion of the DNA polymerase gene comprising amino acids 292 through 834 is selectively amplified in a PCR with forward primer TZA292 and reverse primer DG122 as follows;

30

50 pmoles TZA292
50 pmoles DG122
1 ng <u>EcoRI</u> digested plasmid pLSG22
2.5 units AmpliTaq DNA polymerase
50 μ M each dATP, dGTP, dCTP, dTTP

35

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in an 80 μ l solution containing 10 mM Tris-HCl pH 8.3, 50 mM KCl and overlaid with 100 μ l of mineral oil. The reaction was initiated by addition of 20 μ l containing 7.5 mM MgCl₂ after the tubes had been placed in an 80°C 5 preheated cyclor.

Plasmid pLSG22 (U.S. Serial No. 455,967, filed December 22, 1989, Example 4A, incorporated herein by reference) was digested to completion with restriction 10 endonuclease EcoRI, denatured at 98°C for 5 minutes and cooled rapidly to 0°C. The sample was cycled in a Perkin-Elmer Cetus Thermal Cyclor according to the following profile:

15 STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 55°C and hold for 30 seconds.
RAMP to 72°C over 30 seconds and hold for 1 minute.
REPEAT profile for 3 cycles.

20 STEP CYCLE to 96°C and hold for 20 seconds.
STEP CYCLE to 65°C and hold for 2 minutes.
REPEAT profile for 20 cycles.
After last cycle HOLD for 5 minutes.

25 The intended 1.66 kb PCR product is purified by agarose gel electrophoresis, and recovered following phenol-chloroform extraction and ethanol precipitation. The purified product is digested with restriction endonucleases NdeI and BglII and ligated with 30 NdeI/BamHI-digested and dephosphorylated plasmid vector pDG164 (U.S. Serial No. 455,967, filed December 12, 1989, Example 6B). Ampicillin-resistant transformants of E. coli strain DG116 are selected at 30°C and screened for the desired recombinant plasmid. Plasmid 35 pTTHA292 encodes a 544 amino acid, 5' to 3' exonuclease-deficient Thermus thermophilus thermostable

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DNA polymerase analogous to the pLSG8 encoded protein of Example 2. The DNA polymerase activity is purified as in Example 2. The purified protein is deficient in 5' to 3' exonuclease activity, is more thermoresistant than the corresponding native enzyme and is particularly useful in PCR of G+C-rich templates.

<u>Primer</u>	<u>SEQ ID NO:</u>	<u>SEQUENCE</u>
10 TZA292	SEQ ID NO:32	GTCGGCATATGGCTCCTGCTCCTCTTGAGGA- GGCCCCCTGGCCCCCGCC
DG122	SEQ ID NO:36	CCTCTAAACGGCAGATCTGATATCAACCCTT- GGCGGAAAGC

15

Example 11

20 Derivation and Expression of 5' to 3'
Exonuclease-Deficient, Thermostable DNA
Polymerase of Thermosipho Africanus
Comprising Amino Acids 285 Through 892

To obtain a DNA fragment encoding a 5' to 3' exonuclease-deficient thermostable DNA polymerase from
25 Thermosipho africanus, a portion of the DNA polymerase gene comprising amino acids 285 through 892 is selectively amplified in a PCR with forward primer TAFI285 and reverse primer TAFR01 as follows:

30 50 pmoles TAFI285
50 pmoles TAFR01
1 ng plasmid pBSM:TafRV3' DNA
2.5 units AmpliTaq DNA polymerase
50 μ M each dATP, dGTP, dCTP, dTTP

35

in an 80 μ l solution containing 10 mM Tris-HCl pH 8.3, 50 mM KCl and overlaid with 100 μ l of mineral oil. The

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reaction was initiated by addition of 20 μ l containing 7.5 mM $MgCl_2$ after the tubes had been placed in an 80°C preheated cyclor.

5 Plasmid pBSM:TafRV'3 (obtained as described in CETUS CASE 2583.1, EX 4, p53, incorporated herein by reference) was digested with EcoRI to completion and the DNA was denatured at 98°C for 5 minutes and cooled rapidly to 0°C. The sample was cycled in a
10 Perkin-Elmer Cetus Thermal Cyclor according to the following profile:

STEP CYCLE to 95°C and hold for 30 seconds.
STEP CYCLE to 55°C and hold for 30 seconds.
15 RAMP to 72°C over 30 seconds and hold for 1 minute.
REPEAT profile for 3 cycles.

STEP CYCLE to 95°C and hold for 30 minutes.
STEP CYCLE to 65°C and hold for 2 minutes.
20 REPEAT profile for 20 cycles.
After last cycle HOLD for 5 minutes.

The intended 1.86 kb PCR product is purified by agarose gel electrophoresis, and recovered following
25 phenol-chloroform extraction and ethanol precipitation. The purified product is digested with restriction endonucleases NdeI and BamHI and ligated with NdeI/BamHI-digested and dephosphorylated plasmid vector pDG164 (U.S. Serial No. 455,967, filed December 22,
30 1989, Example 6B). Ampicillin-resistant transformants of E. coli strain DG116 are selected at 30°C and screened for the desired recombinant plasmid. Plasmid pTAFI285 encodes a 609 amino acid, 5' to 3' exonuclease-deficient Thermosipho africanus
35 thermostable DNA polymerase analogous to the pTMA15-encoded protein of Example 3. The DNA

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polymerase activity is purified as in Example 3. The purified protein is deficient in 5' to 3' exonuclease activity, is more thermoresistant than the corresponding native enzyme and is particularly useful in PCR of G+C-rich templates.

<u>Primer</u>	<u>SEQ ID NO:</u>	<u>SEQUENCE</u>
TAFI285	SEQ ID NO:37	GTCGGCATATGATTAAAGAACTTAATTTACA- AGAAAAATTAGAAAAGG
TAFR01	SEQ ID NO:38	CCTTTACCCCAGGATCCTCATTTCCCACTCTT- TTCCATAATAAACAT

15 The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the cell lines deposited, since the deposited embodiment is intended as a single
20 illustration of one aspect of the invention and any cell lines that are functionally equivalent are within the scope of this invention. The deposits of materials therein does not constitute an admission that the written description herein contained is inadequate to
25 enable the practice of any aspect of the invention, including the best mode thereof, nor are the deposits to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in
30 addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Gelfand, David H.
Abramson, Richard D.
- (ii) TITLE OF INVENTION: 5' TO 3' EXONUCLEASE MUTATIONS OF
THERMOSTABLE DNA POLYMERASES
- (iii) NUMBER OF SEQUENCES: 38
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Cetus Corporation
 - (B) STREET: 1400 Fifty-third Street
 - (C) CITY: Emeryville
 - (D) STATE: California
 - (F) ZIP: 94608
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WordPerfect 5.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: WO
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 590,490
 - (B) FILING DATE: 28-SEP-1990
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 590,466
 - (B) FILING DATE: 28-SEP-1990
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 590,213
 - (B) FILING DATE: 28-SEP-1990
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 - (A) APPLICATION NUMBER: US 523,394
 - (B) FILING DATE: 15-MAY-1990
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 - (A) APPLICATION NUMBER: US 143,441
 - (B) FILING DATE: 12-JAN-1988
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 063,509
 - (B) FILING DATE: 17-JUN-1987

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- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 899,241
 - (B) FILING DATE: 22-AUG-1986
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 746,121
 - (B) FILING DATE: 15-AUG-1991
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: WO PCT/US90/07641
 - (B) FILING DATE: 21-DEC-1990
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 585,471
 - (B) FILING DATE: 20-SEP-1990
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 455,611
 - (B) FILING DATE: 22-DEC-1989
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 609,157
 - (B) FILING DATE: 02-NOV-1990
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 557,517
 - (B) FILING DATE: 24-JUL-1990
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Sias Ph.D, Stacey R.
 - (B) REGISTRATION NUMBER: 32,630
 - (C) REFERENCE/DOCKET NUMBER: Case No. 2580
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 415-420-3300
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2499 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Thermus aquaticus*

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2496

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG AGG GGG ATG CTG CCC CTC TTT GAG CCC AAG GGC CGG GTC CTC CTG	48
Met Arg Gly Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu	
1 5 10 15	
GTG GAC GGC CAC CAC CTG GCC TAC CGC ACC TTC CAC GCC CTG AAG GGC	96
Val Asp Gly His His Leu Ala Tyr Arg Thr Phe His Ala Leu Lys Gly	
20 25 30	
CTC ACC ACC AGC CGG GGG GAG CCG GTG CAG GCG GTC TAC GGC TTC GCC	144
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala	
35 40 45	
AAG AGC CTC CTC AAG GCC CTC AAG GAG GAC GGG GAC GCG GTG ATC GTG	192
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Ile Val	
50 55 60	
GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC CAC GAG GCC TAC GGG GGG	240
Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Gly Gly	
65 70 75 80	
TAC AAG GCG GGC CGG GCC CCC ACG CCG GAG GAC TTT CCC CGG CAA CTC	288
Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu	
85 90 95	
GCC CTC ATC AAG GAG CTG GTG GAC CTC CTG GGG CTG GCG CGC CTC GAG	336
Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Ala Arg Leu Glu	
100 105 110	
GTC CCG GGC TAC GAG GCG GAC GAC GTC CTG GCC AGC CTG GCC AAG AAG	384
Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Ser Leu Ala Lys Lys	
115 120 125	
GCG GAA AAG GAG GGC TAC GAG GTC CGC ATC CTC ACC GCC GAC AAA GAC	432
Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Lys Asp	
130 135 140	
CTT TAC CAG CTC CTT TCC GAC CGC ATC CAC GTC CTC CAC CCC GAG GGG	480
Leu Tyr Gln Leu Leu Ser Asp Arg Ile His Val Leu His Pro Glu Gly	
145 150 155 160	

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TAC CTC ATC ACC CCG GCC TGG CTT TGG GAA AAG TAC GGC CTG AGG CCC	528
Tyr Leu Ile Thr Pro Ala Trp Leu Trp Glu Lys Tyr Gly Leu Arg Pro	
165 170 175	
GAC CAG TGG GCC GAC TAC CGG GCC CTG ACC GGG GAC GAG TCC GAC AAC	576
Asp Gln Trp Ala Asp Tyr Arg Ala Leu Thr Gly Asp Glu Ser Asp Asn	
180 185 190	
CTT CCC GGG GTC AAG GGC ATC GGG GAG AAG ACG GCG AGG AAG CTT CTG	624
Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Arg Lys Leu Leu	
195 200 205	
GAG GAG TGG GGG AGC CTG GAA GCC CTC CTC AAG AAC CTG GAC CGG CTG	672
Glu Glu Trp Gly Ser Leu Glu Ala Leu Leu Lys Asn Leu Asp Arg Leu	
210 215 220	
AAG CCC GCC ATC CGG GAG AAG ATC CTG GCC CAC ATG GAC GAT CTG AAG	720
Lys Pro Ala Ile Arg Glu Lys Ile Leu Ala His Met Asp Asp Leu Lys	
225 230 235 240	
CTC TCC TGG GAC CTG GCC AAG GTG CGC ACC GAC CTG CCC CTG GAG GTG	768
Leu Ser Trp Asp Leu Ala Lys Val Arg Thr Asp Leu Pro Leu Glu Val	
245 250 255	
GAC TTC GCC AAA AGG CGG GAG CCC GAC CGG GAG AGG CTT AGG GCC TTT	816
Asp Phe Ala Lys Arg Arg Glu Pro Asp Arg Glu Arg Leu Arg Ala Phe	
260 265 270	
CTG GAG AGG CTT GAG TTT GGC AGC CTC CTC CAC GAG TTC GGC CTT CTG	864
Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu	
275 280 285	
GAA AGC CCC AAG GCC CTG GAG GAG GCC CCC TGG CCC CCG CCG GAA GGG	912
Glu Ser Pro Lys Ala Leu Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly	
290 295 300	
GCC TTC GTG GGC TTT GTG CTT TCC CGC AAG GAG CCC ATG TGG GCC GAT	960
Ala Phe Val Gly Phe Val Leu Ser Arg Lys Glu Pro Met Trp Ala Asp	
305 310 315 320	
CTT CTG GCC CTG GCC GCC GCC AGG GGG GGC CGG GTC CAC CGG GCC CCC	1008
Leu Leu Ala Leu Ala Ala Ala Arg Gly Gly Arg Val His Arg Ala Pro	
325 330 335	

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GAG CCT TAT AAA GCC CTC AGG GAC CTG AAG GAG GCG CGG GGG CTT CTC	1056
Glu Pro Tyr Lys Ala Leu Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu	
340 345 350	
GCC AAA GAC CTG AGC GTT CTG GCC CTG AGG GAA GGC CTT GGC CTC CCG	1104
Ala Lys Asp Leu Ser Val Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro	
355 360 365	
CCC GGC GAC GAC CCC ATG CTC CTC GCC TAC CTC CTG GAC CCT TCC AAC	1152
Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn	
370 375 380	
ACC ACC CCC GAG GGG GTG GCC CGG CGC TAC GGC GGG GAG TGG ACG GAG	1200
Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu	
385 390 395 400	
GAG GCG GGG GAG CGG GCC GCC CTT TCC GAG AGG CTC TTC GCC AAC CTG	1248
Glu Ala Gly Glu Arg Ala Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu	
405 410 415	
TGG GGG AGG CTT GAG GGG GAG GAG AGG CTC CTT TGG CTT TAC CGG GAG	1296
Trp Gly Arg Leu Glu Gly Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu	
420 425 430	
GTG GAG AGG CCC CTT TCC GCT GTC CTG GCC CAC ATG GAG GCC ACG GGG	1344
Val Glu Arg Pro Leu Ser Ala Val Leu Ala His Met Glu Ala Thr Gly	
435 440 445	
GTG CGC CTG GAC GTG GCC TAT CTC AGG GCC TTG TCC CTG GAG GTG GCC	1392
Val Arg Leu Asp Val Ala Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala	
450 455 460	
GAG GAG ATC GCC CGC CTC GAG GCC GAG GTC TTC CGC CTG GCC GGC CAC	1440
Glu Glu Ile Ala Arg Leu Glu Ala Glu Val Phe Arg Leu Ala Gly His	
465 470 475 480	
CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTC CTC TTT GAC	1488
Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp	
485 490 495	
GAG CTA GGG CTT CCC GCC ATC GGC AAG ACG GAG AAG ACC GGC AAG CGC	1536
Glu Leu Gly Leu Pro Ala Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg	
500 505 510	

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TCC ACC AGC GCC GCC GTC CTG GAG GCC CTC CGC GAG GCC CAC CCC ATC	1584
Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile	
515 520 525	
GTG GAG AAG ATC CTG CAG TAC CGG GAG CTC ACC AAG CTG AAG AGC ACC	1632
Val Glu Lys Ile Leu Gln Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr	
530 535 540	
TAC ATT GAC CCC TTG CCG GAC CTC ATC CAC CCC AGG ACG GGC CGC CTC	1680
Tyr Ile Asp Pro Leu Pro Asp Leu Ile His Pro Arg Thr Gly Arg Leu	
545 550 555 560	
CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGC AGG CTA AGT AGC	1728
His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser	
565 570 575	
TCC GAT CCC AAC CTC CAG AAC ATC CCC GTC CGC ACC CCG CTT GGG CAG	1776
Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln	
580 585 590	
AGG ATC CGC CGG GCC TTC ATC GCC GAG GAG GGG TGG CTA TTG GTG GCC	1824
Arg Ile Arg Arg Ala Phe Ile Ala Glu Glu Gly Trp Leu Leu Val Ala	
595 600 605	
CTG GAC TAT AGC CAG ATA GAG CTC AGG GTG CTG GCC CAC CTC TCC GGC	1872
Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly	
610 615 620	
GAC GAG AAC CTG ATC CGG GTC TTC CAG GAG GGG CGG GAC ATC CAC ACG	1920
Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr	
625 630 635 640	
GAG ACC GCC AGC TGG ATG TTC GGC GTC CCC CGG GAG GCC GTG GAC CCC	1968
Glu Thr Ala Ser Trp Met Phe Gly Val Pro Arg Glu Ala Val Asp Pro	
645 650 655	
CTG ATG CGC CGG GCG GCC AAG ACC ATC AAC TTC GGG GTC CTC TAC GGC	2016
Leu Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly	
660 665 670	
ATG TCG GCC CAC CGC CTC TCC CAG GAG CTA GCC ATC CCT TAC GAG GAG	2064
Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu	
675 680 685	

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GCC CAG GCC TTC ATT GAG CGC TAC TTT CAG AGC TTC CCC AAG GTG CGG	2112
Ala Gln Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg	
690 695 700	
GCC TGG ATT GAG AAG ACC CTG GAG GAG GGC AGG AGG CGG GGG TAC GTG	2160
Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val	
705 710 715 720	
GAG ACC CTC TTC GGC CGC CGC CGC TAC GTG CCA GAC CTA GAG GCC CGG	2208
Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg	
725 730 735	
GTG AAG AGC GTG CGG GAG GCG GCC GAG CGC ATG GCC TTC AAC ATG CCC	2256
Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro	
740 745 750	
GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTG GCT ATG GTG AAG CTC	2304
Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu	
755 760 765	
TTC CCC AGG CTG GAG GAA ATG GGG GCC AGG ATG CTC CTT CAG GTC CAC	2352
Phe Pro Arg Leu Glu Glu Met Gly Ala Arg Met Leu Leu Gln Val His	
770 775 780	
GAC GAG CTG GTC CTC GAG GCC CCA AAA GAG AGG GCG GAG GCC GTG GCC	2400
Asp Glu Leu Val Leu Glu Ala Pro Lys Glu Arg Ala Glu Ala Val Ala	
785 790 795 800	
CGG CTG GCC AAG GAG GTC ATG GAG GGG GTG TAT CCC CTG GCC GTG CCC	2448
Arg Leu Ala Lys Glu Val Met Glu Gly Val Tyr Pro Leu Ala Val Pro	
805 810 815	
CTG GAG GTG GAG GTG GGG ATA GGG GAG GAC TGG CTC TCC GCC AAG GAG	2496
Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu	
820 825 830	
TGA	2499

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 832 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Arg	Gly	Met	Leu	Pro	Leu	Phe	Glu	Pro	Lys	Gly	Arg	Val	Leu	Leu	1	5	10	15
Val	Asp	Gly	His	His	Leu	Ala	Tyr	Arg	Thr	Phe	His	Ala	Leu	Lys	Gly	20	25	30	
Leu	Thr	Thr	Ser	Arg	Gly	Glu	Pro	Val	Gln	Ala	Val	Tyr	Gly	Phe	Ala	35	40	45	
Lys	Ser	Leu	Leu	Lys	Ala	Leu	Lys	Glu	Asp	Gly	Asp	Ala	Val	Ile	Val	50	55	60	
Val	Phe	Asp	Ala	Lys	Ala	Pro	Ser	Phe	Arg	His	Glu	Ala	Tyr	Gly	Gly	65	70	75	80
Tyr	Lys	Ala	Gly	Arg	Ala	Pro	Thr	Pro	Glu	Asp	Phe	Pro	Arg	Gln	Leu	85	90	95	
Ala	Leu	Ile	Lys	Glu	Leu	Val	Asp	Leu	Leu	Gly	Leu	Ala	Arg	Leu	Glu	100	105	110	
Val	Pro	Gly	Tyr	Glu	Ala	Asp	Asp	Val	Leu	Ala	Ser	Leu	Ala	Lys	Lys	115	120	125	
Ala	Glu	Lys	Glu	Gly	Tyr	Glu	Val	Arg	Ile	Leu	Thr	Ala	Asp	Lys	Asp	130	135	140	
Leu	Tyr	Gln	Leu	Leu	Ser	Asp	Arg	Ile	His	Val	Leu	His	Pro	Glu	Gly	145	150	155	160
Tyr	Leu	Ile	Thr	Pro	Ala	Trp	Leu	Trp	Glu	Lys	Tyr	Gly	Leu	Arg	Pro	165	170	175	
Asp	Gln	Trp	Ala	Asp	Tyr	Arg	Ala	Leu	Thr	Gly	Asp	Glu	Ser	Asp	Asn	180	185	190	
Leu	Pro	Gly	Val	Lys	Gly	Ile	Gly	Glu	Lys	Thr	Ala	Arg	Lys	Leu	Leu	195	200	205	
Glu	Glu	Trp	Gly	Ser	Leu	Glu	Ala	Leu	Leu	Lys	Asn	Leu	Asp	Arg	Leu	210	215	220	
Lys	Pro	Ala	Ile	Arg	Glu	Lys	Ile	Leu	Ala	His	Met	Asp	Asp	Leu	Lys	225	230	235	240
Leu	Ser	Trp	Asp	Leu	Ala	Lys	Val	Arg	Thr	Asp	Leu	Pro	Leu	Glu	Val	245	250	255	
Asp	Phe	Ala	Lys	Arg	Arg	Glu	Pro	Asp	Arg	Glu	Arg	Leu	Arg	Ala	Phe	260	265	270	

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Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu
 275 280 285

Glu Ser Pro Lys Ala Leu Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly
 290 295 300

Ala Phe Val Gly Phe Val Leu Ser Arg Lys Glu Pro Met Trp Ala Asp
 305 310 315 320

Leu Leu Ala Leu Ala Ala Ala Arg Gly Gly Arg Val His Arg Ala Pro
 325 330 335

Glu Pro Tyr Lys Ala Leu Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu
 340 345 350

Ala Lys Asp Leu Ser Val Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro
 355 360 365

Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn
 370 375 380

Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu
 385 390 395 400

Glu Ala Gly Glu Arg Ala Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu
 405 410 415

Trp Gly Arg Leu Glu Gly Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu
 420 425 430

Val Glu Arg Pro Leu Ser Ala Val Leu Ala His Met Glu Ala Thr Gly
 435 440 445

Val Arg Leu Asp Val Ala Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala
 450 455 460

Glu Glu Ile Ala Arg Leu Glu Ala Glu Val Phe Arg Leu Ala Gly His
 465 470 475 480

Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp
 485 490 495

Glu Leu Gly Leu Pro Ala Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg
 500 505 510

Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His Pro Ile
 515 520 525

Val Glu Lys Ile Leu Gln Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr
 530 535 540

Tyr Ile Asp Pro Leu Pro Asp Leu Ile His Pro Arg Thr Gly Arg Leu
 545 550 555 560

His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser
 565 570 575

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Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln
 580 585 590

Arg Ile Arg Arg Ala Phe Ile Ala Glu Glu Gly Trp Leu Leu Val Ala
 595 600 605

Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly
 610 615 620

Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr
 625 630 635 640

Glu Thr Ala Ser Trp Met Phe Gly Val Pro Arg Glu Ala Val Asp Pro
 645 650 655

Leu Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly
 660 665 670

Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
 675 680 685

Ala Gln Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg
 690 695 700

Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val
 705 710 715 720

Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg
 725 730 735

Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro
 740 745 750

Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu
 755 760 765

Phe Pro Arg Leu Glu Glu Met Gly Ala Arg Met Leu Leu Gln Val His
 770 775 780

Asp Glu Leu Val Leu Glu Ala Pro Lys Glu Arg Ala Glu Ala Val Ala
 785 790 795 800

Arg Leu Ala Lys Glu Val Met Glu Gly Val Tyr Pro Leu Ala Val Pro
 805 810 815

Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu
 820 825 830

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2682 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Thermotoga maritima*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2679

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GCG AGA CTA TTT CTC TTT GAT GGA ACT GCT CTG GCC TAC AGA GCG	48
Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala	
1 5 10 15	
TAC TAT GCG CTC GAT AGA TCG CTT TCT ACT TCC ACC GGC ATT CCC ACA	96
Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr	
20 25 30	
AAC GCC ACA TAC GGT GTG GCG AGG ATG CTG GTG AGA TTC ATC AAA GAC	144
Asn Ala Thr Tyr Gly Val Ala Arg Met Leu Val Arg Phe Ile lys Asp	
35 40 45	
CAT ATC ATT GTC GGA AAA GAC TAC GTT GCT GTG GCT TTC GAC AAA AAA	192
His Ile Ile Val Gly Lys Asp Tyr Val Ala Val Ala Phe Asp lys Lys	
50 55 60	
GCT GCC ACC TTC AGA CAC AAG CTC CTC GAG ACT TAC AAG GCT CAA AGA	240
Ala Ala Thr Phe Arg His Lys Leu Leu Glu Thr Tyr Lys Ala Gln Arg	
65 70 75 80	
CCA AAG ACT CCG GAT CTC CTG ATT CAG CAG CTT CCG TAC ATA AAG AAG	288
Pro Lys Thr Pro Asp Leu Leu Ile Gln Gln Leu Pro Tyr Ile Lys Lys	
85 90 95	
CTG GTC GAA GCC CTT GGA ATG AAA GTG CTG GAG GTA GAA GGA TAC GAA	336
Leu Val Glu Ala Leu Gly Met Lys Val Leu Glu Val Glu Gly Tyr Glu	
100 105 110	

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GCG GAC GAT ATA ATT GCC ACT CTG GCT GTG AAG GGG CTT CCG CTT TTT	384
Ala Asp Asp Ile Ile Ala Thr Leu Ala Val Lys Gly Leu Pro Leu Phe 115 120 125	
GAT GAA ATA TTC ATA GTG ACC GGA GAT AAA GAC ATG CTT CAG CTT GTG	432
Asp Glu Ile Phe Ile Val Thr Gly Asp Lys Asp Met Leu Gln Leu Val 130 135 140	
AAC GAA AAG ATC AAG GTG TGG CGA ATC GTA AAA GGG ATA TCC GAT CTG	480
Asn Glu Lys Ile Lys Val Trp Arg Ile Val Lys Gly Ile Ser Asp Leu 145 150 155 160	
GAA CTT TAC GAT GCG CAG AAG GTG AAG GAA AAA TAC GGT GTT GAA CCC	528
Glu Leu Tyr Asp Ala Gln Lys Val Lys Glu Lys Tyr Gly Val Glu Pro 165 170 175	
CAG CAG ATC CCG GAT CTT CTG GCT CTA ACC GGA GAT GAA ATA GAC AAC	576
Gln Gln Ile Pro Asp Leu Leu Ala Leu Thr Gly Asp Glu Ile Asp Asn 180 185 190	
ATC CCC GGT GTA ACT GGG ATA GGT GAA AAG ACT GCT GTT CAG CTT CTA	624
Ile Pro Gly Val Thr Gly Ile Gly Glu Lys Thr Ala Val Gln Leu Leu 195 200 205	
GAG AAG TAC AAA GAC CTC GAA GAC ATA CTG AAT CAT GTT CGC GAA CTT	672
Glu Lys Tyr Lys Asp Leu Glu Asp Ile Leu Asn His Val Arg Glu Leu 210 215 220	
CCT CAA AAG GTG AGA AAA GCC CTG CTT CGA GAC AGA GAA AAC GCC ATT	720
Pro Gln Lys Val Arg Lys Ala Leu Leu Arg Asp Arg Glu Asn Ala Ile 225 230 235 240	
CTC AGC AAA AAG CTG GCG ATT CTG GAA ACA AAC GTT CCC ATT GAA ATA	768
Leu Ser Lys Lys Leu Ala Ile Leu Glu Thr Asn Val Pro Ile Glu Ile 245 250 255	
AAC TGG GAA GAA CTT CGC TAC CAG GGC TAC GAC AGA GAG AAA CTC TTA	816
Asn Trp Glu Glu Leu Arg Tyr Gln Gly Tyr Asp Arg Glu Lys Leu Leu 260 265 270	
CCA CTT TTG AAA GAA CTG GAA TTC GCA TCC ATC ATG AAG GAA CTT CAA	864
Pro Leu Leu Lys Glu Leu Glu Phe Ala Ser Ile Met Lys Glu leu Gln 275 280 285	

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CTG TAC GAA GAG TCC GAA CCC GTT GGA TAC AGA ATA GTG AAA GAC CTA	912
Leu Tyr Glu Glu Ser Glu Pro Val Gly Tyr Arg Ile Val Lys Asp Leu	
290 295 300	
GTG GAA TTT GAA AAA CTC ATA GAG AAA CTG AGA GAA TCC CCT TCG TTC	960
Val Glu Phe Glu Lys Leu Ile Glu Lys Leu Arg Glu Ser Pro Ser Phe	
305 310 315 320	
GCC ATA GAT CTT GAG ACG TCT TCC CTC GAT CCT TTC GAC TGC GAC ATT	1008
Ala Ile Asp Leu Glu Thr Ser Ser Leu Asp Pro Phe Asp Cys Asp Ile	
325 330 335	
GTC GGT ATC TCT GTG TCT TTC AAA CCA AAG GAA GCG TAC TAC ATA CCA	1056
Val Gly Ile Ser Val Ser Phe Lys Pro Lys Glu Ala Tyr Tyr Ile Pro	
340 345 350	
CTC CAT CAT AGA AAC GCC CAG AAC CTG GAC GAA AAA GAG GTT CTG AAA	1104
Leu His His Arg Asn Ala Gln Asn Leu Asp Glu Lys Glu Val Leu Lys	
355 360 365	
AAG CTC AAA GAA ATT CTG GAG GAC CCC GGA GCA AAG ATC GTT GGT CAG	1152
Lys Leu Lys Glu Ile Leu Glu Asp Pro Gly Ala Lys Ile Val Gly Gln	
370 375 380	
AAT TTG AAA TTC GAT TAC AAG GTG TTG ATG GTG AAG GGT GTT GAA CCT	1200
Asn Leu Lys Phe Asp Tyr Lys Val Leu Met Val Lys Gly Val Glu Pro	
385 390 395 400	
GTT CCT CCT TAC TTC GAC ACG ATG ATA GCG GCT TAC CTT CTT GAG CCG	1248
Val Pro Pro Tyr Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro	
405 410 415	
AAC GAA AAG AAG TTC AAT CTG GAC GAT CTC GCA TTG AAA TTT CTT GGA	1296
Asn Glu Lys Lys Phe Asn Leu Asp Asp Leu Ala Leu Lys Phe Leu Gly	
420 425 430	
TAC AAA ATG ACA TCT TAC GAA GAG CTC ATG TCC TTC TCT TTT CCG CTG	1344
Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Phe Pro Leu	
435 440 445	
TTT GGT TTC AGT TTT GCC GAT GTT CCT GTA GAA AAA GCA GCG AAC TAC	1392
Phe Gly Phe Ser Phe Ala Asp Val Pro Val Glu Lys Ala Ala Asn Tyr	
450 455 460	

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TCG TGT GAA GAT GCA GAC ATC ACC TAC AGA CTT TAC AAG ACC CTG AGC	1440
Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Thr Leu Ser	
465 470 475 480	
TTA AAA CTC CAC GAG GCA GAT CTG GAA AAC GTG TTC TAC AAG ATA GAA	1488
Leu Lys Leu His Glu Ala Asp Leu Glu Asn Val Phe Tyr Lys Ile Glu	
485 490 495	
ATG CCC CTT GTG AAC GTG CTT GCA CGG ATG GAA CTG AAC GGT GTG TAT	1536
Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr	
500 505 510	
GTG GAC ACA GAG TTC CTG AAG AAA CTC TCA GAA GAG TAC GGA AAA AAA	1584
Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys	
515 520 525	
CTC GAA GAA CTG GCA GAG GAA ATA TAC AGG ATA GCT GGA GAG CCG TTC	1632
Leu Glu Glu Leu Ala Glu Glu Ile Tyr Arg Ile Ala Gly Glu Pro Phe	
530 535 540	
AAC ATA AAC TCA CCG AAG CAG GTT TCA AGG ATC CTT TTT GAA AAA CTC	1680
Asn Ile Asn Ser Pro Lys Gln Val Ser Arg Ile Leu Phe Glu Lys Leu	
545 550 555 560	
GGC ATA AAA CCA CGT GGT AAA ACG ACG AAA ACG GGA GAC TAT TCA ACA	1728
Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Asp Tyr Ser Thr	
565 570 575	
CGC ATA GAA GTC CTC GAG GAA CTT GCC GGT GAA CAC GAA ATC ATT CCT	1776
Arg Ile Glu Val Leu Glu Glu Leu Ala Gly Glu His Glu Ile Ile Pro	
580 585 590	
CTG ATT CTT GAA TAC AGA AAG ATA CAG AAA TTG AAA TCA ACC TAC ATA	1824
Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr Ile	
595 600 605	
GAC GCT CTT CCC AAG ATG GTC AAC CCA AAG ACC GGA AGG ATT CAT GCT	1872
Asp Ala Leu Pro Lys Met Val Asn Pro Lys Thr Gly Arg Ile His Ala	
610 615 620	
TCT TTC AAT CAA ACG GGG ACT GCC ACT GGA AGA CTT AGC AGC AGC GAT	1920
Ser Phe Asn Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp	
625 630 635 640	

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CCC AAT CTT CAG AAC CTC CCG ACG AAA AGT GAA GAG GGA AAA GAA ATC	1968
Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile	
645 650 655	
AGG AAA GCG ATA GTT CCT CAG GAT CCA AAC TGG TGG ATC GTC AGT GCC	2016
Arg Lys Ala Ile Val Pro Gln Asp Pro Asn Trp Trp Ile Val Ser Ala	
660 665 670	
GAC TAC TCC CAA ATA GAA CTG AGG ATC CTC GCC CAT CTC AGT GGT GAT	2064
Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp	
675 680 685	
GAG AAT CTT TTG AGG GCA TTC GAA GAG GGC ATC GAC GTC CAC ACT CTA	2112
Glu Asn Leu Leu Arg Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu	
690 695 700	
ACA GCT TCC AGA ATA TTC AAC GTG AAA CCC GAA GAA GTA ACC GAA GAA	2160
Thr Ala Ser Arg Ile Phe Asn Val Lys Pro Glu Glu Val Thr Glu Glu	
705 710 715 720	
ATG CGC CGC GCT GGT AAA ATG GTT AAT TTT TCC ATC ATA TAC GGT GTA	2208
Met Arg Arg Ala Gly Lys Met Val Asn Phe Ser Ile Ile Tyr Gly Val	
725 730 735	
ACA CCT TAC GGT CTG TCT GTG AGG CTT GGA GTA CCT GTG AAA GAA GCA	2256
Thr Pro Tyr Gly Leu Ser Val Arg Leu Gly Val Pro Val Lys Glu Ala	
740 745 750	
GAA AAG ATG ATC GTC AAC TAC TTC GTC CTC TAC CCA AAG GTG CGC GAT	2304
Glu Lys Met Ile Val Asn Tyr Phe Val Leu Tyr Pro Lys Val Arg Asp	
755 760 765	
TAC ATT CAG AGG GTC GTA TCG GAA GCG AAA GAA AAA GGC TAT GTT AGA	2352
Tyr Ile Gln Arg Val Val Ser Glu Ala Lys Glu Lys Gly Tyr Val Arg	
770 775 780	
ACG CTG TTT GGA AGA AAA AGA GAC ATA CCA CAG CTC ATG GCC CCG GAC	2400
Thr Leu Phe Gly Arg Lys Arg Asp Ile Pro Gln Leu Met Ala Arg Asp	
785 790 795 800	
AGG AAC ACA CAG GCT GAA GGA GAA CGA ATT GCC ATA AAC ACT CCC ATA	2448
Arg Asn Thr Gln Ala Glu Gly Glu Arg Ile Ala Ile Asn Thr Pro Ile	
805 810 815	

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CAG GGT ACA GCA GCG GAT ATA ATA AAG CTG GCT ATG ATA GAA ATA GAC 2496
 Gln Gly Thr Ala Ala Asp Ile Ile Lys Leu Ala Met Ile Glu Ile Asp
 820 825 830
 AGG GAA CTG AAA GAA AGA AAA ATG AGA TCG AAG ATG ATC ATA CAG GTC 2544
 Arg Glu Leu Lys Glu Arg Lys Met Arg Ser Lys Met Ile Ile Gln Val
 835 840 845
 CAC GAC GAA CTG GTT TTT GAA GTG CCC AAT GAG GAA AAG GAC GCG CTC 2592
 His Asp Glu Leu Val Phe Glu Val Pro Asn Glu Glu Lys Asp Ala Leu
 850 855 860
 GTC GAG CTG GTG AAA GAC AGA ATG ACG AAT GTG GTA AAG CTT TCA GTG 2640
 Val Glu Leu Val Lys Asp Arg Met Thr Asn Val Val Lys Leu Ser Val
 865 870 875 880
 CCG CTC GAA GTG GAT GTA ACC ATC GGC AAA ACA TGG TCG TGA 2682
 Pro Leu Glu Val Asp Val Thr Ile Gly Lys Thr Trp Ser
 885 890

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 893 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala
 1 5 10 15
 Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr
 20 25 30
 Asn Ala Thr Tyr Gly Val Ala Arg Met Leu Val Arg Phe Ile Lys Asp
 35 40 45
 His Ile Ile Val Gly Lys Asp Tyr Val Ala Val Ala Phe Asp Lys Lys
 50 55 60
 Ala Ala Thr Phe Arg His Lys Leu Leu Glu Thr Tyr Lys Ala Gln Arg
 65 70 75 80
 Pro Lys Thr Pro Asp Leu Leu Ile Gln Gln Leu Pro Tyr Ile Lys Lys
 85 90 95

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Leu Val Glu Ala Leu Gly Met Lys Val Leu Glu Val Glu Gly Tyr Glu
 100 105 110

Ala Asp Asp Ile Ile Ala Thr Leu Ala Val Lys Gly Leu Pro Leu Phe
 115 120 125

Asp Glu Ile Phe Ile Val Thr Gly Asp Lys Asp Met Leu Gln Leu Val
 130 135 140

Asn Glu Lys Ile Lys Val Trp Arg Ile Val Lys Gly Ile Ser Asp Leu
 145 150 155 160

Glu Leu Tyr Asp Ala Gln Lys Val Lys Glu Lys Tyr Gly Val Glu Pro
 165 170 175

Gln Gln Ile Pro Asp Leu Leu Ala Leu Thr Gly Asp Glu Ile Asp Asn
 180 185 190

Ile Pro Gly Val Thr Gly Ile Gly Glu Lys Thr Ala Val Gln Leu Leu
 195 200 205

Glu Lys Tyr Lys Asp Leu Glu Asp Ile Leu Asn His Val Arg Glu Leu
 210 215 220

Pro Gln Lys Val Arg Lys Ala Leu Leu Arg Asp Arg Glu Asn Ala Ile
 225 230 235 240

Leu Ser Lys Lys Leu Ala Ile Leu Glu Thr Asn Val Pro Ile Glu Ile
 245 250 255

Asn Trp Glu Glu Leu Arg Tyr Gln Gly Tyr Asp Arg Glu Lys Leu Leu
 260 265 270

Pro Leu Leu Lys Glu Leu Glu Phe Ala Ser Ile Met Lys Glu Leu Gln
 275 280 285

Leu Tyr Glu Glu Ser Glu Pro Val Gly Tyr Arg Ile Val Lys Asp Leu
 290 295 300

Val Glu Phe Glu Lys Leu Ile Glu Lys Leu Arg Glu Ser Pro Ser Phe
 305 310 315 320

Ala Ile Asp Leu Glu Thr Ser Ser Leu Asp Pro Phe Asp Cys Asp Ile
 325 330 335

Val Gly Ile Ser Val Ser Phe Lys Pro Lys Glu Ala Tyr Tyr Ile Pro
 340 345 350

Leu His His Arg Asn Ala Gln Asn Leu Asp Glu Lys Glu Val Leu Lys
 355 360 365

Lys Leu Lys Glu Ile Leu Glu Asp Pro Gly Ala Lys Ile Val Gly Gln
 370 375 380

Asn Leu Lys Phe Asp Tyr Lys Val Leu Met Val Lys Gly Val Glu Pro
 385 390 395 400

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Val Pro Pro Tyr Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro
 405 410 415
 Asn Glu Lys Lys Phe Asn Leu Asp Asp Leu Ala Leu Lys Phe Leu Gly
 420 425 430
 Tyr Lys Met Thr Ser Tyr Gln Glu Leu Met Ser Phe Ser Phe Pro Leu
 435 440 445
 Phe Gly Phe Ser Phe Ala Asp Val Pro Val Glu Lys Ala Ala Asn Tyr
 450 455 460
 Ser Cys Glu Asp Ala Asp Ile Thr Tyr Arg Leu Tyr Lys Thr Leu Ser
 465 470 475 480
 Leu Lys Leu His Glu Ala Asp Leu Glu Asn Val Phe Tyr Lys Ile Glu
 485 490 495
 Met Pro Leu Val Asn Val Leu Ala Arg Met Glu Leu Asn Gly Val Tyr
 500 505 510
 Val Asp Thr Glu Phe Leu Lys Lys Leu Ser Glu Glu Tyr Gly Lys Lys
 515 520 525
 Leu Glu Glu Leu Ala Glu Glu Ile Tyr Arg Ile Ala Gly Glu Pro Phe
 530 535 540
 Asn Ile Asn Ser Pro Lys Gln Val Ser Arg Ile Leu Phe Glu Lys Leu
 545 550 555 560
 Gly Ile Lys Pro Arg Gly Lys Thr Thr Lys Thr Gly Asp Tyr Ser Thr
 565 570 575
 Arg Ile Glu Val Leu Glu Glu Leu Ala Gly Glu His Glu Ile Ile Pro
 580 585 590
 Leu Ile Leu Glu Tyr Arg Lys Ile Gln Lys Leu Lys Ser Thr Tyr Ile
 595 600 605
 Asp Ala Leu Pro Lys Met Val Asn Pro Lys Thr Gly Arg Ile His Ala
 610 615 620
 Ser Phe Asn Gln Thr Gly Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp
 625 630 635 640
 Pro Asn Leu Gln Asn Leu Pro Thr Lys Ser Glu Glu Gly Lys Glu Ile
 645 650 655
 Arg Lys Ala Ile Val Pro Gln Asp Pro Asn Trp Trp Ile Val Ser Ala
 660 665 670
 Asp Tyr Ser Gln Ile Glu Leu Arg Ile Leu Ala His Leu Ser Gly Asp
 675 680 685
 Glu Asn Leu Leu Arg Ala Phe Glu Glu Gly Ile Asp Val His Thr Leu
 690 695 700

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Thr	Ala	Ser	Arg	Ile	Phe	Asn	Val	Lys	Pro	Glu	Glu	Val	Thr	Glu	Glu	705	710	715	720
Met	Arg	Arg	Ala	Gly	Lys	Met	Val	Asn	Phe	Ser	Ile	Ile	Tyr	Gly	Val	725	730	735	
Thr	Pro	Tyr	Gly	Leu	Ser	Val	Arg	Leu	Gly	Val	Pro	Val	Lys	Glu	Ala	740	745	750	
Glu	Lys	Met	Ile	Val	Asn	Tyr	Phe	Val	Leu	Tyr	Pro	Lys	Val	Arg	Asp	755	760	765	
Tyr	Ile	Gln	Arg	Val	Val	Ser	Glu	Ala	Lys	Glu	Lys	Gly	Tyr	Val	Arg	770	775	780	
Thr	Leu	Phe	Gly	Arg	Lys	Arg	Asp	Ile	Pro	Gln	Leu	Met	Ala	Arg	Asp	785	790	795	800
Arg	Asn	Thr	Gln	Ala	Glu	Gly	Glu	Arg	Ile	Ala	Ile	Asn	Thr	Pro	Ile	805	810	815	
Gln	Gly	Thr	Ala	Ala	Asp	Ile	Ile	Lys	Leu	Ala	Met	Ile	Glu	Ile	Asp	820	825	830	
Arg	Glu	Leu	Lys	Glu	Arg	Lys	Met	Arg	Ser	Lys	Met	Ile	Ile	Gln	Val	835	840	845	
His	Asp	Glu	Leu	Val	Phe	Glu	Val	Pro	Asn	Glu	Glu	Lys	Asp	Ala	Leu	850	855	860	
Val	Glu	Leu	Val	Lys	Asp	Arg	Met	Thr	Asn	Val	Val	Lys	Leu	Ser	Val	865	870	875	880
Pro	Leu	Glu	Val	Asp	Val	Thr	Ile	Gly	Lys	Thr	Trp	Ser				885	890		

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2493 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:

- (A) ORGANISM: *Thermus species sps17*

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2490

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG CTG CCC CTC TTT GAG CCC AAG GGC CGG GTC CTC CTG GTG GAC GGC	48
Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu Val Asp Gly	
1 5 10 15	
CAC CAC CTG GCC TAC CGC ACC TTT TTC GCC CTC AAG GGC CTC ACC ACC	96
His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly Leu Thr Thr	
20 25 30	
AGC CGG GGC GAG CCC GTG CAG GCG GTT TAT GGC TTC GCC AAA AGC CTC	144
Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala Lys Ser Leu	
35 40 45	
CTC AAG GCC CTG AAG GAG GAT GGG GAG GTG GCC ATC GTG GTC TTT GAC	192
Leu Lys Ala Leu Lys Glu Asp Gly Glu Val Ala Ile Val Val Phe Asp	
50 55 60	
GCC AAG GCC CCC TCC TTC CGC CAG GAG GCC TAC GAG GCC TAC AAG GCG	240
Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu Ala Tyr Lys Ala	
65 70 75 80	
GGC CGG GCC CCC ACC CCG GAG GAC TTT CCC CGG CAG CTC GCC CTC ATC	288
Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu Ala Leu Ile	
85 90 95	
AAG GAG CTG GTG GAC CTT TTG GGC CTC GTG CGC CTT GAG GTC CCG GGC	336
Lys Glu Leu Val Asp Leu Leu Gly Leu Val Arg Leu Glu Val Pro Gly	
100 105 110	
TTT GAG GCG GAC GAT GTC CTC GCC ACC CTG GCC AAG AAG GCA GAA AGG	384
Phe Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys Lys Ala Clu Arg	
115 120 125	
GAG GGG TAC GAG GTG CGC ATC CTG AGC GCG GAC CGC GAC CTC TAC CAG	432
Glu Gly Tyr Glu Val Arg Ile Leu Ser Ala Asp Arg Asp Leu Tyr Gln	
130 135 140	
CTC CTT TCC GAC CGG ATC CAC CTC CTC CAC CCC GAG GGG GAG GTC CTC	480
Leu Leu Ser Asp Arg Ile His Leu Leu His Pro Glu Gly Glu Val Leu	
145 150 155 160	

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ACC CCC GGG TGG CTC CAG GAG CGC TAC GGC CTC TCC CCG GAG AGG TGG	528
Thr Pro Gly Trp Leu Gln Glu Arg Tyr Gly Leu Ser Pro Glu Arg Trp	
165 170 175	
GTG GAG TAC CGG GCC CTG GTG GGG GAC CCT TCG GAC AAC CTC CCC GGG	576
Val Glu Tyr Arg Ala Leu Val Gly Asp Pro Ser Asp Asn Leu Pro Gly	
180 185 190	
GTG CCC GGC ATC GGG GAG AAG ACC GCC CTG AAG CTC CTG AAG GAG TGG	624
Val Pro Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu Leu Lys Glu Trp	
195 200 205	
GGT AGC CTG GAA GCG ATT CTA AAG AAC CTG GAC CAG GTG AAG CCG GAA	672
Gly Ser Leu Glu Ala Ile Leu Lys Asn Leu Asp Gln Val Lys Pro Glu	
210 215 220	
AGG GTG CGG GAG GCC ATC CGG AAT AAC CTG GAT AAG CTC CAG ATG TCC	720
Arg Val Arg Glu Ala Ile Arg Asn Asn Leu Asp Lys Leu Gln Met Ser	
225 230 235 240	
CTG GAG CTT TCC CGC CTC CGC ACC GAC CTC CCC CTG GAG GTG GAC TTC	768
Leu Glu Leu Ser Arg Leu Arg Thr Asp Leu Pro Leu Glu Val Asp Phe	
245 250 255	
GCC AAG AGG CGG GAG CCC GAC TGG GAG GGG CTT AAG GCC TTT TTG GAG	816
Ala Lys Arg Arg Glu Pro Asp Trp Glu Gly Leu Lys Ala Phe Leu Glu	
260 265 270	
CGG CTT GAG TTC GGA AGC CTC CTC CAC GAG TTC GGC CTT CTG GAG GCC	864
Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu Clu Ala	
275 280 285	
CCC AAG GAG GCG GAG GAG GCC CCC TGG CCC CCG CCT GGA GGG GCC TTT	912
Pro Lys Glu Ala Glu Glu Ala Pro Trp Pro Pro Pro Gly Gly Ala Phe	
290 295 300	
TTG GGC TTC CTC CTC TCC CGC CCC GAG CCC ATG TGG GCG GAG CTT TTG	960
Leu Gly Phe Leu Leu Ser Arg Pro Glu Pro Met Trp Ala Glu Leu Leu	
305 310 315 320	
GCC CTG GCG GGG GCC AAG GAG GGG CGG GTC CAT CGG GCG GAA GAC CCC	1008
Ala Leu Ala Gly Ala Lys Glu Gly Arg Val His Arg Ala Glu Asp Pro	
325 330 335	

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GTG GGG GCC CTA AAG GAC CTG AAG GAG ATC CGG GGC CTC CTC GCC AAG	1056
Val Gly Ala Leu Lys Asp Leu Lys Glu Ile Arg Gly Leu Leu Ala Lys	
340 345 350	
GAC CTC TCG GTC CTG GCC CTG AGG GAG GGC CGG GAG ATC CCG CCG GGG	1104
Asp Leu Ser Val Leu Ala Leu Arg Glu Gly Arg Glu Ile Pro Pro Gly	
355 360 365	
GAC GAC CCC ATG CTC CTC GCC TAC CTC CTG GAC CCG GGG AAC ACC AAC	1152
Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Gly Asn Thr Asn	
370 375 380	
CCC GAG GGG GTG GCC CGG CGG TAC GGG GGG GAG TGG AAG GAG GAC GCC	1200
Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Lys Glu Asp Ala	
385 390 395 400	
GCC GCC CGG GCC CTC CTT TCG GAA AGG CTC TGG CAG GCC CTT TAC CCC	1248
Ala Ala Arg Ala Leu Leu Ser Glu Arg Leu Trp Gln Ala Leu Tyr Pro	
405 410 415	
CGG GTG GCG GAG GAG GAA AGG CTC CTT TGG CTC TAC CGG GAG GTG GAG	1296
Arg Val Ala Glu Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu	
420 425 430	
CGG CCC CTC GCC CAG GTC CTC GCC CAC ATG GAG GCC ACG GGG GTG CGG	1344
Arg Pro Leu Ala Gln Val Leu Ala His Met Glu Ala Thr Gly Val Arg	
435 440 445	
CTG GAT GTG CCC TAC CTG GAG GCC CTT TCC CAG GAG GTG GCC TTT GAG	1392
Leu Asp Val Pro Tyr Leu Glu Ala Leu Ser Gln Glu Val Ala Phe Glu	
450 455 460	
CTG GAG CGC CTC GAG GCC GAG GTC CAC CGC CTG GCG GGC CAC CCC TTC	1440
Leu Glu Arg Leu Glu Ala Glu Val His Arg Leu Ala Gly His Pro Phe	
465 470 475 480	
AAC CTG AAC TCT AGG GAC CAG CTG GAG CGG GTC CTC TTT GAC GAG CTC	1488
Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu	
485 490 495	
GGC CTA CCC CCC ATC GGC AAG ACG GAG AAG ACG GGC AAG CGC TCC ACC	1536
Gly Leu Pro Pro Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr	
500 505 510	

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AGC GCC GCC GTC CTG GAG CTC TTA AGG GAG GCC CAC CCC ATC GTG GGG	1584
Ser Ala Ala Val Leu Glu Leu Leu Arg Glu Ala His Pro Ile Val Gly	
515 520 525	
CGG ATC CTG GAG TAC CGG GAG CTC ATG AAG CTC AAG AGC ACC TAC ATA	1632
Arg Ile Leu Glu Tyr Arg Glu Leu Met Lys Leu Lys Ser Thr Tyr Ile	
530 535 540	
GAC CCC CTC CCC AGG CTG GTC CAC CCC AAA ACC GGC CGG CTC CAC ACC	1680
Asp Pro Leu Pro Arg Leu Val His Pro Lys Thr Gly Arg Leu His Thr	
545 550 555 560	
CGC TTC AAC CAG ACG GCC ACC GCC ACG GGC CGC CTC TCC AGC TCC GAC	1728
Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp	
565 570 575	
CCC AAC CTG CAG AAC ATC CCC GTG CGC ACC CCC TTA GGC CAG CGC ATC	1776
Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg Ile	
580 585 590	
CGC AAG GCC TTC ATT GCC GAG GAG GGC CAT CTC CTG GTG GCC CTG GAC	1824
Arg Lys Ala Phe Ile Ala Glu Glu Gly His Leu Leu Val Ala Leu Asp	
595 600 605	
TAT AGC CAG ATC GAG CTC CGG GTC CTC GCC CAC CTC TCG GGG GAC GAG	1872
Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly Asp Glu	
610 615 620	
AAC CTC ATC CGG GTC TTC CGG GAA GGG AAG GAC ATC CAC ACC GAG ACC	1920
Asn Leu Ile Arg Val Phe Arg Glu Gly Lys Asp Ile His Thr Glu Thr	
625 630 635 640	
GCC GCC TGG ATG TTC GGC GTG CCC CCC GAG GGG GTG GAC GGG GCC ATG	1968
Ala Ala Trp Met Phe Gly Val Pro Pro Glu Gly Val Asp Gly Ala Met	
645 650 655	
CGC CGG GCG GCC AAG ACG GTG AAC TTC GGG GTG CTC TAC GGG ATG TCC	2016
Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu Tyr Gly Met Ser	
660 665 670	
GCC CAC CGC CTC TCC CAG GAG CTC TCC ATC CCC TAC GAG GAG GCG GCG	2064
Ala His Arg Leu Ser Gln Glu Leu Ser Ile Pro Tyr Glu Glu Ala Ala	
675 680 685	

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GCC TTC ATC GAG CGC TAC TTC CAG AGC TTC CCC AAG GTG CGG GCC TGG	2112
Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg Ala Trp	
690 695 700	
ATC GCC AAA ACC TTG GAG GAG GGG CGG AAG AAG GGG TAC GTG GAG ACC	2160
Ile Ala Lys Thr Leu Glu Glu Gly Arg Lys Lys Gly Tyr Val Glu Thr	
705 710 715 720	
CTC TTC GGC CGC CGC CGC TAC GTG CCC GAC CTC AAC GCC CGG GTG AAG	2208
Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn Ala Arg Val Lys	
725 730 735	
AGC GTG CGG GAG GCG GCG GAG CGC ATG GCC TTC AAC ATG CCC GTG CAG	2256
Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro Val Gln	
740 745 750	
GGC ACC GCC GCG GAC CTC ATG AAG CTG GCC ATG GTG AAG CTC TTC CCC	2304
Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu Phe Pro	
755 760 765	
AGG CTC AGG CCC TTG GGC GTT CGC ATC CTC CTC CAG GTG CAC GAC GAG	2352
Arg Leu Arg Pro Leu Gly Val Arg Ile Leu Leu Gln Val His Asp Glu	
770 775 780	
CTG GTC TTG GAG GCC CCA AAG GCG CGG GCG GAG GAG GCC GCC CAG TTG	2400
Leu Val Leu Glu Ala Pro Lys Ala Arg Ala Glu Glu Ala Ala Gln Leu	
785 790 795 800	
GCC AAG GAG ACC ATG GAA GGG GTT TAC CCC CTC TCC GTC CCC CTG GAG	2448
Ala Lys Glu Thr Met Glu Gly Val Tyr Pro Leu Ser Val Pro Leu Glu	
805 810 815	
GTG GAG GTG GGG ATG GGG GAG GAC TGG CTT TCC GCC AAG GCC	2490
Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala Lys Ala	
820 825 830	
TAG	2493

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 830 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu Val Asp Gly
 1           5           10           15
His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly Leu Thr Thr
          20           25           30
Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala Lys Ser Leu
          35           40           45
Leu Lys Ala Leu Lys Glu Asp Gly Glu Val Ala Ile Val Val Phe Asp
          50           55           60
Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu Ala Tyr Lys Ala
          65           70           75           80
Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu Ala Leu Ile
          85           90           95
Lys Glu Leu Val Asp Leu Leu Gly Leu Val Arg Leu Glu Val Pro Gly
          100          105          110
Phe Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys Lys Ala Glu Arg
          115          120          125
Glu Gly Tyr Glu Val Arg Ile Leu Ser Ala Asp Arg Asp Leu Tyr Gln
          130          135          140
Leu Leu Ser Asp Arg Ile His Leu Leu His Pro Glu Gly Glu Val Leu
          145          150          155          160
Thr Pro Gly Trp Leu Gln Glu Arg Tyr Gly Leu Ser Pro Glu Arg Trp
          165          170          175
Val Glu Tyr Arg Ala Leu Val Gly Asp Pro Ser Asp Asn Leu Pro Gly
          180          185          190
Val Pro Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu Leu Lys Glu Trp
          195          200          205
Gly Ser Leu Glu Ala Ile Leu Lys Asn Leu Asp Gln Val Lys Pro Glu
          210          215          220
Arg Val Arg Glu Ala Ile Arg Asn Asn Leu Asp Lys Leu Gln Met Ser
          225          230          235          240
Leu Glu Leu Ser Arg Leu Arg Thr Asp Leu Pro Leu Glu Val Asp Phe
          245          250          255
Ala Lys Arg Arg Glu Pro Asp Trp Glu Gly Leu Lys Ala Phe Leu Glu
          260          265          270

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Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly Leu Leu Glu Ala
 275 280 285
 Pro Lys Glu Ala Glu Glu Ala Pro Trp Pro Pro Pro Gly Gly Ala Phe
 290 295 300
 Leu Gly Phe Leu Leu Ser Arg Pro Glu Pro Met Trp Ala Glu Leu Leu
 305 310 315 320
 Ala Leu Ala Gly Ala Lys Glu Gly Arg Val His Arg Ala Glu Asp Pro
 325 330 335
 Val Gly Ala Leu Lys Asp Leu Lys Glu Ile Arg Gly Leu Leu Ala Lys
 340 345 350
 Asp Leu Ser Val Leu Ala Leu Arg Glu Gly Arg Glu Ile Pro Pro Gly
 355 360 365
 Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro Gly Asn Thr Asn
 370 375 380
 Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp Lys Glu Asp Ala
 385 390 395 400
 Ala Ala Arg Ala Leu Leu Ser Glu Arg Leu Trp Gln Ala Leu Tyr Pro
 405 410 415
 Arg Val Ala Glu Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu
 420 425 430
 Arg Pro Leu Ala Gln Val Leu Ala His Met Glu Ala Thr Gly Val Arg
 435 440 445
 Leu Asp Val Pro Tyr Leu Glu Ala Leu Ser Gln Glu Val Ala Phe Glu
 450 455 460
 Leu Glu Arg Leu Glu Ala Glu Val His Arg Leu Ala Gly His Pro Phe
 465 470 475 480
 Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu
 485 490 495
 Gly Leu Pro Pro Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr
 500 505 510
 Ser Ala Ala Val Leu Glu Leu Leu Arg Glu Ala His Pro Ile Val Gly
 515 520 525
 Arg Ile Leu Glu Tyr Arg Glu Leu Met Lys Leu Lys Ser Thr Tyr Ile
 530 535 540
 Asp Pro Leu Pro Arg Leu Val His Pro Lys Thr Gly Arg Leu His Thr
 545 550 555 560
 Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp
 565 570 575

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Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg Ile
 580 585 590
 Arg Lys Ala Phe Ile Ala Glu Glu Gly His Leu Leu Val Ala Leu Asp
 595 600 605
 Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly Asp Glu
 610 615 620
 Asn Leu Ile Arg Val Phe Arg Glu Gly Lys Asp Ile His Thr Glu Thr
 625 630 635 640
 Ala Ala Trp Met Phe Gly Val Pro Pro Glu Gly Val Asp Gly Ala Met
 645 650 655
 Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu Tyr Gly Met Ser
 660 665 670
 Ala His Arg Leu Ser Gln Glu Leu Ser Ile Pro Tyr Glu Glu Ala Ala
 675 680 685
 Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg Ala Trp
 690 695 700
 Ile Ala Lys Thr Leu Glu Glu Gly Arg Lys Lys Gly Tyr Val Glu Thr
 705 710 715 720
 Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn Ala Arg Val Lys
 725 730 735
 Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro Val Gln
 740 745 750
 Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu Phe Pro
 755 760 765
 Arg Leu Arg Pro Leu Gly Val Arg Ile Leu Leu Gln Val His Asp Glu
 770 775 780
 Leu Val Leu Glu Ala Pro Lys Ala Arg Ala Glu Glu Ala Ala Gln Leu
 785 790 795 800
 Ala Lys Glu Thr Met Glu Gly Val Tyr Pro Leu Ser Val Pro Leu Glu
 805 810 815
 Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala Lys Ala
 820 825 830

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2505 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Thermus species* Z05

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2502

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG AAG GCG ATG CTT CCG CTC TTT GAA CCC AAA GGC CGG GTT CTC CTG	48
Met Lys Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu	
1 5 10 15	
GTG GAC GGC CAC CAC CTG GCC TAC CGC ACC TTC TTC GCC CTA AAG GGC	96
Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly	
20 25 30	
CTC ACC ACG AGC CGG GGC GAA CCG GTG CAG GCG GTT TAC GGC TTC GCC	144
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala	
35 40 45	
AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC AAG GCC GTC TTC	192
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe	
50 55 60	
GTG GTC TTT GAC GCC AAG GCC CCT TCC TTC CGC CAC GAG GCC TAC GAG	240
Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu	
65 70 75 80	
GCC TAC AAG GCA GGC CGC GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG	288
Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln	
85 90 95	
CTC GCC CTC ATC AAG GAG CTG GTG GAC CTC CTG GGG TTT ACT CGC CTC	336
Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu	
100 105 110	

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GAG GTT CCG GGC TTT GAG GCG GAC GAC GTC CTC GCC ACC CTG GCC AAG	384
Glu Val Pro Gly Phe Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys	
115 120 125	
AAG GCG GAA AGG GAG GGG TAC GAG GTG CGC ATC CTC ACC GCC GAC CGG	432
Lys Ala Glu Arg Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg	
130 135 140	
GAC CTT TAC CAG CTC GTC TCC GAC CGC GTC GCC GTC CTC CAC CCC GAG	480
Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu	
145 150 155 160	
GGC CAC CTC ATC ACC CCG GAG TGG CTT TGG GAG AAG TAC GGC CTT AAG	528
Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Lys	
165 170 175	
CCG GAG CAG TGG GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC	576
Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp	
180 185 190	
AAC CTC CCC GGG GTC AAG GGC ATC GGG GAG AAG ACC GCC CTC AAG CTC	624
Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu	
195 200 205	
CTC AAG GAG TGG GGA AGC CTG GAA AAT ATC CTC AAG AAC CTG GAC CGG	672
Leu Lys Glu Trp Gly Ser Leu Glu Asn Ile Leu Lys Asn Leu Asp Arg	
210 215 220	
GTG AAG CCG GAA AGC GTC CGG GAA AGG ATC AAG GCC CAC CTG GAA GAC	720
Val Lys Pro Glu Ser Val Arg Glu Arg Ile Lys Ala His Leu Clu Asp	
225 230 235 240	
CTT AAG CTC TCC TTG GAG CTT TCC CGG GTG CGC TCG GAC CTC CCC CTG	768
Leu Lys Leu Ser Leu Glu Leu Ser Arg Val Arg Ser Asp Leu Pro Leu	
245 250 255	
GAG GTG GAC TTC GCC CGG AGG CGG GAG CCT GAC CGG GAA GGG CTT CGG	816
Glu Val Asp Phe Ala Arg Arg Arg Glu Pro Asp Arg Glu Gly Leu Arg	
260 265 270	
GCC TTT TTG GAG CGC TTG GAG TTC GGC AGC CTC CTC CAC GAG TTC GGC	864
Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly	
275 280 285	

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CTC CTC GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC TGG CCC CCG CCG	912
Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro 290 295 300	
GAA GGG GCC TTC GTG GCC TTC GTC CTC TCC CGC CCC GAG CCC ATG TGG	960
Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp 305 310 315 320	
GCG GAG CTT AAA GCC CTG GCC GCC TGC AAG GAG GGC CGG GTG CAC CGG	1008
Ala Glu Leu Lys Ala Leu Ala Ala Cys Lys Glu Gly Arg Val His Arg 325 330 335	
GCA AAG GAC CCC TTG GCG GGG CTA AAG GAC CTC AAG GAG GTC CGA GGC	1056
Ala Lys Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly 340 345 350	
CTC CTC GCC AAG GAC CTC GCC GTT TTG GCC CTT CGC GAG GGG CTG GAC	1104
Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Leu Arg Glu Gly Leu Asp 355 360 365	
CTC GCG CCT TCG GAC GAC CCC ATG CTC CTC GCC TAC CTC CTG GAC CCC	1152
Leu Ala Pro Ser Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro 370 375 380	
TCC AAC ACC ACC CCC GAG GGG GTG GCC CGG CGC TAC GGG GGG GAG TGG	1200
Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp 385 390 395 400	
ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC GCC GAG CGG CTC CAG CAA	1248
Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ala Glu Arg Leu Gln Gln 405 410 415	
AAC CTC TTG GAA CGC CTC AAG GGA GAG GAA AAG CTC CTT TGG CTC TAC	1296
Asn Leu Leu Glu Arg Leu Lys Gly Glu Glu Lys Leu Leu Trp Leu Tyr 420 425 430	
CAA GAG GTG GAA AAG CCC CTC TCC CGG GTC CTG GCC CAC ATG GAG GCC	1344
Gln Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala 435 440 445	
ACC GGG GTA AGG CTG GAC GTG GCC TAT CTA AAG GCC CTT TCC CTG GAG	1392
Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Lys Ala Leu Ser Leu Glu 450 455 460	

CTT GCG GAG GAG ATT CGC CGC CTC GAG GAG GAG GTC TTC CGC CTG GCG	1440
Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala 465 470 475 480	
GGC CAC CCC TTC AAC CTG AAC TCC CGT GAC CAG CTA GAG CGG GTG CTC	1488
Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu 485 490 495	
TTT GAC GAG CTT AGG CTT CCC GCC CTG GGC AAG ACG CAA AAG ACG GGG	1536
Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly 500 505 510	
AAG CGC TCC ACC AGC GCC GCG GTG CTG GAG GCC CTC AGG GAG GCC CAC	1584
Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His 515 520 525	
CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC ACC AAG CTC AAG	1632
Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys 530 535 540	
AAC ACC TAC GTG GAC CCC CTC CCG GGC CTC GTC CAC CCG AGG ACG GGC	1680
Asn Thr Tyr Val Asp Pro Leu Pro Gly Leu Val His Pro Arg Thr Gly 545 550 555 560	
CGC CTC CAC ACC CGC TTC AAC CAG ACA GCC ACG GCC ACG GGA AGG CTC	1728
Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu 565 570 575	
TCT AGC TCC GAC CCC AAC CTG CAG AAC ATC CCC ATC CGC ACC CCC TTG	1776
Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Ile Arg Thr Pro Leu 580 585 590	
GGC CAG AGG ATC CGC CGG GCC TTC GTG GCC GAG GCG GGA TGG GCG TTG	1824
Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu 595 600 605	
GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGG GTC CTC GCC CAC CTC	1872
Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu 610 615 620	
TCC GGG GAC GAG AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC	1920
Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile 625 630 635 640	

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CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC TCC CCG GAG GCC GTG	1968
His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Ser Pro Glu Ala Val	
645 650 655	
GAC CCC CTG ATG CGC CGG GCG GCC AAG ACG GTG AAC TTC GGC GTC CTC	2016
Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu	
660 665 670	
TAC GGC ATG TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC ATC CCC TAC	2064
Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr	
675 680 685	
GAG GAG GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA AGC TTC CCC AAG	2112
Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys	
690 695 700	
GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC	2160
Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly	
705 710 715 720	
TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC AAC	2208
Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn	
725 730 735	
GCC CGG GTG AAG AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC	2256
Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn	
740 745 750	
ATG CCC GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTC GCC ATG GTG	2304
Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val	
755 760 765	
AAG CTC TTC CCC CAC CTC CGG GAG ATG GGG GCC CGC ATG CTC CTC CAG	2352
Lys Leu Phe Pro His Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln	
770 775 780	
GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG GCC GAG GAG	2400
Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu	
785 790 795 800	
GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC	2448
Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala	
805 810 815	

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GTG CCC CTG GAG GTG GAG GTG GGG ATC GGG GAG GAC TGG CTT TCC GCC 2496
 Val Pro Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala
 820 825 830

AAG GGC TGA 2505
 Lys Gly

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 834 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
 1 5 10 15

Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
 20 25 30

Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
 35 40 45

Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
 50 55 60

Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
 65 70 75 80

Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
 85 90 95

Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
 100 105 110

Glu Val Pro Gly Phe Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
 115 120 125

Lys Ala Glu Arg Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
 130 135 140

Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu
 145 150 155 160

Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Lys
 165 170 175

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Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
 180 185 190
 Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
 195 200 205
 Leu Lys Glu Trp Gly Ser Leu Glu Asn Ile Leu Lys Asn Leu Asp Arg
 210 215 220
 Val Lys Pro Glu Ser Val Arg Glu Arg Ile Lys Ala His Leu Glu Asp
 225 230 235 240
 Leu Lys Leu Ser Leu Glu Leu Ser Arg Val Arg Ser Asp Leu Pro Leu
 245 250 255
 Glu Val Asp Phe Ala Arg Arg Arg Glu Pro Asp Arg Glu Gly Leu Arg
 260 265 270
 Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly
 275 280 285
 Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro
 290 295 300
 Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp
 305 310 315 320
 Ala Glu Leu Lys Ala Leu Ala Ala Cys Lys Glu Gly Arg Val His Arg
 325 330 335
 Ala Lys Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly
 340 345 350
 Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Leu Arg Glu Gly Leu Asp
 355 360 365
 Leu Ala Pro Ser Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro
 370 375 380
 Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp
 385 390 395 400
 Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ala Glu Arg Leu Gln Gln
 405 410 415
 Asn Leu Leu Glu Arg Leu Lys Gly Glu Glu Lys Leu Leu Trp Leu Tyr
 420 425 430
 Gln Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala
 435 440 445
 Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Lys Ala Leu Ser Leu Glu
 450 455 460
 Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala
 465 470 475 480

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Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu
 485 490 495
 Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly
 500 505 510
 Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His
 515 520 525
 Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys
 530 535 540
 Asn Thr Tyr Val Asp Pro Leu Pro Gly Leu Val His Pro Arg Thr Gly
 545 550 555 560
 Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu
 565 570 575
 Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Ile Arg Thr Pro Leu
 580 585 590
 Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu
 595 600 605
 Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu
 610 615 620
 Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile
 625 630 635 640
 His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Ser Pro Glu Ala Val
 645 650 655
 Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu
 660 665 670
 Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr
 675 680 685
 Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys
 690 695 700
 Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly
 705 710 715 720
 Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn
 725 730 735
 Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn
 740 745 750
 Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val
 755 760 765
 Lys Leu Phe Pro His Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
 770 775 780

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Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
 785 790 795 800

Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
 805 810 815

Val Pro Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala
 820 825 830

Lys Gly

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2505 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Thermus thermophilus*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2502

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG GAG GCG ATG CTT CCG CTC TTT GAA CCC AAA GGC CGG GTC CTC CTG	48
Met Glu Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu	
1 5 10 15	
GTG GAC GGC CAC CAC CTG GCC TAC CGC ACC TTC TTC GCC CTG AAG GGC	96
Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly	
20 25 30	
CTC ACC ACG AGC CGG GGC GAA CCG GTG CAG GCG GTC TAC GGC TTC GCC	144
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala	
35 40 45	
AAG AGC CTC CTC AAG GCC CTG AAG GAG GAC GGG TAC AAG GCC GTC TTC	192
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe	
50 55 60	

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GTG GTC TTT GAC GCC AAG GCC CCC TCC TTC CGC CAC GAG GCC TAC GAG	240
Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu 65 70 75 80	
GCC TAC AAG GCG GGG AGG GCC CCG ACC CCC GAG GAC TTC CCC CGG CAG	288
Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln 85 90 95	
CTC GCC CTC ATC AAG GAG CTG GTG GAC CTC CTG GGG TTT ACC CGC CTC	336
Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu 100 105 110	
GAG GTC CCC GGC TAC GAG GCG GAC GAC GTT CTC GCC ACC CTG GCC AAG	384
Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys 115 120 125	
AAG GCG GAA AAG GAG GGG TAC GAG GTG CGC ATC CTC ACC GCC GAC CGC	432
Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg 130 135 140	
GAC CTC TAC CAA CTC GTC TCC GAC CGC GTC GCC GTC CTC CAC CCC GAG	480
Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu 145 150 155 160	
GGC CAC CTC ATC ACC CCG GAG TGG CTT TGG GAG AAG TAC GGC CTC AGG	528
Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Arg 165 170 175	
CCG GAG CAG TGG GTG GAC TTC CGC GCC CTC GTG GGG GAC CCC TCC GAC	576
Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp 180 185 190	
AAC CTC CCC GGG GTC AAG GGC ATC GGG GAG AAG ACC GCC CTC AAG CTC	624
Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu 195 200 205	
CTC AAG GAG TGG GGA AGC CTG GAA AAC CTC CTC AAG AAC CTG GAC CGG	672
Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg 210 215 220	
GTA AAG CCA GAA AAC GTC CGG GAG AAG ATC AAG GCC CAC CTG GAA GAC	720
Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp 225 230 235 240	

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CTC AGG CTC TCC TTG GAG CTC TCC CGG GTG CGC ACC GAC CTC CCC CTG	768
Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu	
245 250 255	
GAG GTG GAC CTC GCC CAG GGG CGG GAG CCC GAC CGG GAG GGG CTT AGG	816
Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg	
260 265 270	
GCC TTC CTG GAG AGG CTG GAG TTC GGC AGC CTC CTC CAC GAG TTC GGC	864
Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly	
275 280 285	
CTC CTG GAG GCC CCC GCC CCC CTG GAG GAG GCC CCC TGG CCC CCG CCG	912
Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro	
290 295 300	
GAA GGG GCC TTC GTG GGC TTC GTC CTC TCC CGC CCC GAG CCC ATG TGG	960
Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp	
305 310 315 320	
GCG GAG CTT AAA GCC CTG GCC GCC TGC AGG GAC GGC CGG GTG CAC CGG	1008
Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg	
325 330 335	
GCA GCA GAC CCC TTG GCG GGG CTA AAG GAC CTC AAG GAG GTC CGG GGC	1056
Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly	
340 345 350	
CTC CTC GCC AAG GAC CTC GCC GTC TTG GCC TCG AGG GAG GGG CTA GAC	1104
Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp	
355 360 365	
CTC GTG CCC GGG GAC GAC CCC ATG CTC CTC GCC TAC CTC CTG GAC CCC	1152
Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro	
370 375 380	
TCC AAC ACC ACC CCC GAG GGG GTG GCG CGG CGC TAC GGG GGG GAG TGG	1200
Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp	
385 390 395 400	
ACG GAG GAC GCC GCC CAC CGG GCC CTC CTC TCG GAG AGG CTC CAT CGG	1248
Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg	
405 410 415	

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AAC CTC CTT AAG CGC CTC GAG GGG GAG GAG AAG CTC CTT TGG CTC TAC	1296
Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr	
420 425 430	
CAC GAG GTG GAA AAG CCC CTC TCC CGG GTC CTG GCC CAC ATG GAG GCC	1344
His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala	
435 440 445	
ACC GGG GTA CGG CTG GAC GTG GCC TAC CTT CAG GCC CTT TCC CTG GAG	1392
Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu	
450 455 460	
CTT GCG GAG GAG ATC CGC CGC CTC GAG GAG GAG GTC TTC CGC TTG GCG	1440
Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala	
465 470 475 480	
GGC CAC CCC TTC AAC CTC AAC TCC CGG GAC CAG CTG GAA AGG GTG CTC	1488
Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu	
485 490 495	
TTT GAC GAG CTT AGG CTT CCC GCC TTG GGG AAG ACG CAA AAG ACA GGC	1536
Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly	
500 505 510	
AAG CGC TCC ACC AGC GCC GCG GTG CTG GAG GCC CTA CGG GAG GCC CAC	1584
Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His	
515 520 525	
CCC ATC GTG GAG AAG ATC CTC CAG CAC CGG GAG CTC ACC AAG CTC AAG	1632
Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys	
530 535 540	
AAC ACC TAC GTG GAC CCC CTC CCA AGC CTC GTC CAC CCG AGG ACG GGC	1680
Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly	
545 550 555 560	
CGC CTC CAC ACC CGC TTC AAC CAG ACG GCC ACG GCC ACG GGG AGG CTT	1728
Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu	
565 570 575	
AGT AGC TCC GAC CCC AAC CTG CAG AAC ATC CCC GTC CGC ACC CCC TTG	1776
Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu	
580 585 590	

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GGC CAG AGG ATC CGC CGG GCC TTC GTG GCC GAG GCG GGT TGG GCG TTG	1824
Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu	
595 600 605	
GTG GCC CTG GAC TAT AGC CAG ATA GAG CTC CGC GTC CTC GCC CAC CTC	1872
Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu	
610 615 620	
TCC GGG GAC GAA AAC CTG ATC AGG GTC TTC CAG GAG GGG AAG GAC ATC	1920
Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile	
625 630 635 640	
CAC ACC CAG ACC GCA AGC TGG ATG TTC GGC GTC CCC CCG GAG GCC GTG	1968
His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val	
645 650 655	
GAC CCC CTG ATG CGC CGG GCG GCC AAG ACG GTG AAC TTC GGC GTC CTC	2016
Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu	
660 665 670	
TAC GGC ATG TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC ATC CCC TAC	2064
Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr	
675 680 685	
GAG GAG GCG GTG GCC TTT ATA GAG CGC TAC TTC CAA AGC TTC CCC AAG	2112
Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys	
690 695 700	
GTG CGG GCC TGG ATA GAA AAG ACC CTG GAG GAG GGG AGG AAG CGG GGC	2160
Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly	
705 710 715 720	
TAC GTG GAA ACC CTC TTC GGA AGA AGG CGC TAC GTG CCC GAC CTC AAC	2208
Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn	
725 730 735	
GCC CGG GTG AAG AGC GTC AGG GAG GCC GCG GAG CGC ATG GCC TTC AAC	2256
Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn	
740 745 750	
ATG CCC GTC CAG GGC ACC GCC GCC GAC CTC ATG AAG CTC GCC ATG GTG	2304
Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val	
755 760 765	

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AAG CTC TTC CCC CGC CTC CGG GAG ATG GGG GCC CGC ATG CTC CTC CAG 2352
 Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
 770 775 780
 GTC CAC GAC GAG CTC CTC CTG GAG GCC CCC CAA GCG CGG GCC GAG GAG 2400
 Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
 785 790 795 800
 GTG GCG GCT TTG GCC AAG GAG GCC ATG GAG AAG GCC TAT CCC CTC GCC 2448
 Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
 805 810 815
 GTG CCC CTG GAG GTG GAG GTG GGG ATG GGG GAG GAC TGG CTT TCC GCC 2496
 Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala
 820 825 830
 AAG GGT TAG 2505
 Lys Gly

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 834 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Glu Ala Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
 1 5 10 15
 Val Asp Gly His His Leu Ala Tyr Arg Thr Phe Phe Ala Leu Lys Gly
 20 25 30
 Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
 35 40 45
 Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Tyr Lys Ala Val Phe
 50 55 60
 Val Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Glu
 65 70 75 80
 Ala Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln
 85 90 95

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Leu Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Phe Thr Arg Leu
 100 105 110
 Glu Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Thr Leu Ala Lys
 115 120 125
 Lys Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Arg
 130 135 140
 Asp Leu Tyr Gln Leu Val Ser Asp Arg Val Ala Val Leu His Pro Glu
 145 150 155 160
 Gly His Leu Ile Thr Pro Glu Trp Leu Trp Glu Lys Tyr Gly Leu Arg
 165 170 175
 Pro Glu Gln Trp Val Asp Phe Arg Ala Leu Val Gly Asp Pro Ser Asp
 180 185 190
 Asn Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Leu Lys Leu
 195 200 205
 Leu Lys Glu Trp Gly Ser Leu Glu Asn Leu Leu Lys Asn Leu Asp Arg
 210 215 220
 Val Lys Pro Glu Asn Val Arg Glu Lys Ile Lys Ala His Leu Glu Asp
 225 230 235 240
 Leu Arg Leu Ser Leu Glu Leu Ser Arg Val Arg Thr Asp Leu Pro Leu
 245 250 255
 Glu Val Asp Leu Ala Gln Gly Arg Glu Pro Asp Arg Glu Gly Leu Arg
 260 265 270
 Ala Phe Leu Glu Arg Leu Glu Phe Gly Ser Leu Leu His Glu Phe Gly
 275 280 285
 Leu Leu Glu Ala Pro Ala Pro Leu Glu Glu Ala Pro Trp Pro Pro Pro
 290 295 300
 Glu Gly Ala Phe Val Gly Phe Val Leu Ser Arg Pro Glu Pro Met Trp
 305 310 315 320
 Ala Glu Leu Lys Ala Leu Ala Ala Cys Arg Asp Gly Arg Val His Arg
 325 330 335
 Ala Ala Asp Pro Leu Ala Gly Leu Lys Asp Leu Lys Glu Val Arg Gly
 340 345 350
 Leu Leu Ala Lys Asp Leu Ala Val Leu Ala Ser Arg Glu Gly Leu Asp
 355 360 365
 Leu Val Pro Gly Asp Asp Pro Met Leu Leu Ala Tyr Leu Leu Asp Pro
 370 375 380
 Ser Asn Thr Thr Pro Glu Gly Val Ala Arg Arg Tyr Gly Gly Glu Trp
 385 390 395 400

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Thr Glu Asp Ala Ala His Arg Ala Leu Leu Ser Glu Arg Leu His Arg
 405 410 415
 Asn Leu Leu Lys Arg Leu Glu Gly Glu Glu Lys Leu Leu Trp Leu Tyr
 420 425 430
 His Glu Val Glu Lys Pro Leu Ser Arg Val Leu Ala His Met Glu Ala
 435 440 445
 Thr Gly Val Arg Leu Asp Val Ala Tyr Leu Gln Ala Leu Ser Leu Glu
 450 455 460
 Leu Ala Glu Glu Ile Arg Arg Leu Glu Glu Glu Val Phe Arg Leu Ala
 465 470 475 480
 Gly His Pro Phe Asn Leu Asn Ser Arg Asp Gln Leu Glu Arg Val Leu
 485 490 495
 Phe Asp Glu Leu Arg Leu Pro Ala Leu Gly Lys Thr Gln Lys Thr Gly
 500 505 510
 Lys Arg Ser Thr Ser Ala Ala Val Leu Glu Ala Leu Arg Glu Ala His
 515 520 525
 Pro Ile Val Glu Lys Ile Leu Gln His Arg Glu Leu Thr Lys Leu Lys
 530 535 540
 Asn Thr Tyr Val Asp Pro Leu Pro Ser Leu Val His Pro Arg Thr Gly
 545 550 555 560
 Arg Leu His Thr Arg Phe Asn Gln Thr Ala Thr Ala Thr Gly Arg Leu
 565 570 575
 Ser Ser Ser Asp Pro Asn Leu Gln Asn Ile Pro Val Arg Thr Pro Leu
 580 585 590
 Gly Gln Arg Ile Arg Arg Ala Phe Val Ala Glu Ala Gly Trp Ala Leu
 595 600 605
 Val Ala Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu
 610 615 620
 Ser Gly Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Lys Asp Ile
 625 630 635 640
 His Thr Gln Thr Ala Ser Trp Met Phe Gly Val Pro Pro Glu Ala Val
 645 650 655
 Asp Pro Leu Met Arg Arg Ala Ala Lys Thr Val Asn Phe Gly Val Leu
 660 665 670
 Tyr Gly Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr
 675 680 685
 Glu Glu Ala Val Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys
 690 695 700

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Val Arg Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Lys Arg Gly
 705 710 715 720
 Tyr Val Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Asn
 725 730 735
 Ala Arg Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn
 740 745 750
 Met Pro Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val
 755 760 765
 Lys Leu Phe Pro Arg Leu Arg Glu Met Gly Ala Arg Met Leu Leu Gln
 770 775 780
 Val His Asp Glu Leu Leu Leu Glu Ala Pro Gln Ala Arg Ala Glu Glu
 785 790 795 800
 Val Ala Ala Leu Ala Lys Glu Ala Met Glu Lys Ala Tyr Pro Leu Ala
 805 810 815
 Val Pro Leu Glu Val Glu Val Gly Met Gly Glu Asp Trp Leu Ser Ala
 820 825 830
 Lys Gly

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2679 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Thermosipho africanus*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2676

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATG GGA AAG ATG TTT CTA TTT GAT GGA ACT GGA TTA GTA TAC AGA GCA 48
 Met Gly Lys Met Phe Leu Phe Asp Gly Thr Gly Leu Val Tyr Arg Ala
 1 5 10 15

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TTT TAT GCT ATA GAT CAA TCT CTT CAA ACT TCG TCT GGT TTA CAC ACT	96
Phe Tyr Ala Ile Asp Gln Ser Leu Gln Thr Ser Ser Gly Leu His Thr	
20 25 30	
AAT GCT GTA TAC GGA CTT ACT AAA ATG CTT ATA AAA TTT TTA AAA GAA	144
Asn Ala Val Tyr Gly Leu Thr Lys Met Leu Ile Lys Phe Leu Lys Glu	
35 40 45	
CAT ATC AGT ATT GGA AAA GAT GCT TGT GTT TTT GTT TTA GAT TCA AAA	192
His Ile Ser Ile Gly Lys Asp Ala Cys Val Phe Val Leu Asp Ser Lys	
50 55 60	
GGT GGT AGC AAA AAA AGA AAG GAT ATT CTT GAA ACA TAT AAA GCA AAT	240
Gly Gly Ser Lys Lys Arg Lys Asp Ile Leu Glu Thr Tyr Lys Ala Asn	
65 70 75 80	
AGG CCA TCA ACG CCT GAT TTA CTT TTA GAG CAA ATT CCA TAT GTA GAA	288
Arg Pro Ser Thr Pro Asp Leu Leu Leu Glu Gln Ile Pro Tyr Val Glu	
85 90 95	
GAA CTT GTT GAT GCT CTT GGA ATA AAA GTT TTA AAA ATA GAA GGC TTT	336
Glu Leu Val Asp Ala Leu Gly Ile Lys Val Leu Lys Ile Glu Gly Phe	
100 105 110	
GAA GCT GAT GAC ATT ATT GCT ACG CTT TCT AAA AAA TTT GAA AGT GAT	384
Glu Ala Asp Asp Ile Ile Ala Thr Leu Ser Lys Lys Phe Glu Ser Asp	
115 120 125	
TTT GAA AAG GTA AAC ATA ATA ACT GGA GAT AAA GAT CTT TTA CAA CTT	432
Phe Glu Lys Val Asn Ile Ile Thr Gly Asp Lys Asp Leu Leu Cln Leu	
130 135 140	
GTT TCT GAT AAG GTT TTT GTT TGG AGA GTA GAA AGA GGA ATA ACA GAT	480
Val Ser Asp Lys Val Phe Val Trp Arg Val Glu Arg Gly Ile Thr Asp	
145 150 155 160	
TTG GTA TTG TAC GAT AGA AAT AAA GTG ATT GAA AAA TAT GGA ATC TAC	528
Leu Val Leu Tyr Asp Arg Asn Lys Val Ile Glu Lys Tyr Gly Ile Tyr	
165 170 175	
CCA GAA CAA TTC AAA GAT TAT TTA TCT CTT GTC GGT GAT CAG ATT GAT	576
Pro Glu Gln Phe Lys Asp Tyr Leu Ser Leu Val Gly Asp Gln Ile Asp	
180 185 190	

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AAT ATC CCA GGA GTT AAA GGA ATA GGA AAG AAA ACA GCT GTT TCG CTT	624
Asn Ile Pro Gly Val Lys Gly Ile Gly Lys Lys Thr Ala Val Ser Leu	
195 200 205	
TTG AAA AAA TAT AAT AGC TTG GAA AAT GTA TTA AAA AAT ATT AAC CTT	672
Leu Lys Lys Tyr Asn Ser Leu Glu Asn Val Leu Lys Asn Ile Asn Leu	
210 215 220	
TTG ACG GAA AAA TTA AGA AGG CTT TTG GAA GAT TCA AAG GAA GAT TTG	720
Leu Thr Glu Lys Leu Arg Arg Leu Leu Glu Asp Ser Lys Glu Asp Leu	
225 230 235 240	
CAA AAA AGT ATA GAA CTT GTG GAG TTG ATA TAT GAT GTA CCA ATG GAT	768
Gln Lys Ser Ile Glu Leu Val Glu Leu Ile Tyr Asp Val Pro Met Asp	
245 250 255	
GTG GAA AAA GAT GAA ATA ATT TAT AGA GGG TAT AAT CCA GAT AAG CTT	816
Val Glu Lys Asp Glu Ile Ile Tyr Arg Gly Tyr Asn Pro Asp Lys Leu	
260 265 270	
TTA AAG GTA TTA AAA AAG TAC GAA TTT TCA TCT ATA ATT AAG GAG TTA	864
Leu Lys Val Leu Lys Lys Tyr Glu Phe Ser Ser Ile Ile Lys Glu Leu	
275 280 285	
AAT TTA CAA GAA AAA TTA GAA AAG GAA TAT ATA CTG GTA GAT AAT GAA	912
Asn Leu Gln Glu Lys Leu Glu Lys Glu Tyr Ile Leu Val Asp Asn Glu	
290 295 300	
GAT AAA TTG AAA AAA CTT GCA GAA GAG ATA GAA AAA TAC AAA ACT TTT	960
Asp Lys Leu Lys Lys Leu Ala Glu Glu Ile Glu Lys Tyr Lys Thr Phe	
305 310 315 320	
TCA ATT GAT ACG GAA ACA ACT TCA CTT GAT CCA TTT GAA GCT AAA CTG	1008
Ser Ile Asp Thr Glu Thr Thr Ser Leu Asp Pro Phe Glu Ala Lys Leu	
325 330 335	
GTT GGG ATC TCT ATT TCG ACA ATG GAA GGG AAG GCG TAT TAT ATT CCG	1056
Val Gly Ile Ser Ile Ser Thr Met Glu Gly Lys Ala Tyr Tyr Ile Pro	
340 345 350	
GTG TCT CAT TTT GGA GCT AAG AAT ATT TCC AAA AGT TTA ATA GAT AAA	1104
Val Ser His Phe Gly Ala Lys Asn Ile Ser Lys Ser Leu Ile Asp Lys	
355 360 365	

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TTT CTA AAA CAA ATT TTG CAA GAG AAG GAT TAT AAT ATC GTT GGT CAG	1152
Phe Leu Lys Gln Ile Leu Gln Glu Lys Asp Tyr Asn Ile Val Gly Gln	
370 375 380	
AAT TTA AAA TTT GAC TAT GAG ATT TTT AAA AGC ATG GGT TTT TCT CCA	1200
Asn Leu Lys Phe Asp Tyr Glu Ile Phe Lys Ser Met Gly Phe Ser Pro	
385 390 395 400	
AAT GTT CCG CAT TTT GAT ACG ATG ATT GCA GCC TAT CTT TTA AAT CCA	1248
Asn Val Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Asn Pro	
405 410 415	
GAT GAA AAA CGT TTT AAT CTT GAA GAG CTA TCG TTA AAA TAT TTA GGT	1296
Asp Glu Lys Arg Phe Asn Leu Glu Glu Leu Ser Leu Lys Tyr Leu Gly	
420 425 430	
TAT AAA ATG ATC TCG TTT GAT GAA TTA GTA AAT GAA AAT GTA CCA TTG	1344
Tyr Lys Met Ile Ser Phe Asp Glu Leu Val Asn Glu Asn Val Pro Leu	
435 440 445	
TTT GGA AAT GAC TTT TCG TAT GTT CCA CTA GAA AGA GCC GTT GAG TAT	1392
Phe Gly Asn Asp Phe Ser Tyr Val Pro Leu Glu Arg Ala Val Glu Tyr	
450 455 460	
TCC TGT GAA GAT GCC GAT GTG ACA TAC AGA ATA TTT AGA AAG CTT GGT	1440
Ser Cys Glu Asp Ala Asp Val Thr Tyr Arg Ile Phe Arg Lys Leu Gly	
465 470 475 480	
AGG AAG ATA TAT GAA AAT GAG ATG GAA AAG TTG TTT TAC GAA ATT GAG	1488
Arg Lys Ile Tyr Glu Asn Glu Met Glu Lys Leu Phe Tyr Glu Ile Glu	
485 490 495	
ATG CCC TTA ATT GAT GTT CTT TCA GAA ATG GAA CTA AAT GGA GTG TAT	1536
Met Pro Leu Ile Asp Val Leu Ser Glu Met Glu Leu Asn Gly Val Tyr	
500 505 510	
TTT GAT GAG GAA TAT TTA AAA GAA TTA TCA AAA AAA TAT CAA GAA AAA	1584
Phe Asp Glu Glu Tyr Leu Lys Glu Leu Ser Lys Lys Tyr Gln Glu Lys	
515 520 525	
ATG GAT GGA ATT AAG GAA AAA GTT TTT GAG ATA GCT GGT GAA ACT TTC	1632
Met Asp Gly Ile Lys Glu Lys Val Phe Glu Ile Ala Gly Glu Thr Phe	
530 535 540	

AAT TTA AAC TCT TCA ACT CAA GTA GCA TAT ATA CTA TTT GAA AAA TTA	1680
Asn Leu Asn Ser Ser Thr Gln Val Ala Tyr Ile Leu Phe Glu Lys Leu 545 550 555 560	
AAT ATT GCT CCT TAC AAA AAA ACA GCG ACT GGT AAG TTT TCA ACT AAT	1728
Asn Ile Ala Pro Tyr Lys Lys Thr Ala Thr Gly Lys Phe Ser Thr Asn 565 570 575	
GCG GAA GTT TTA GAA GAA CTT TCA AAA GAA CAT GAA ATT GCA AAA TTG	1776
Ala Glu Val Leu Glu Glu Leu Ser Lys Glu His Glu Ile Ala Lys Leu 580 585 590	
TTG CTG GAG TAT CGA AAG TAT CAA AAA TTA AAA AGT ACA TAT ATT GAT	1824
Leu Leu Glu Tyr Arg Lys Tyr Gln Lys Leu Lys Ser Thr Tyr Ile Asp 595 600 605	
TCA ATA CCG TTA TCT ATT AAT CGA AAA ACA AAC AGG GTC CAT ACT ACT	1872
Ser Ile Pro Leu Ser Ile Asn Arg Lys Thr Asn Arg Val His Thr Thr 610 615 620	
TTT CAT CAA ACA GGA ACT TCT ACT GGA AGA TTA AGT AGT TCA AAT CCA	1920
Phe His Gln Thr Gly Thr Ser Thr Gly Arg Leu Ser Ser Ser Asn Pro 625 630 635 640	
AAT TTG CAA AAT CTT CCA ACA AGA AGC GAA GAA GGA AAA GAA ATA AGA	1968
Asn Leu Gln Asn Leu Pro Thr Arg Ser Glu Glu Gly Lys Glu Ile Arg 645 650 655	
AAA GCA GTA AGA CCT CAA AGA CAA GAT TGG TGG ATT TTA GGT GCT GAC	2016
Lys Ala Val Arg Pro Gln Arg Gln Asp Trp Trp Ile Leu Gly Ala Asp 660 665 670	
TAT TCT CAG ATA GAA CTA AGG GTT TTA GCG CAT GTA AGT AAA GAT GAA	2064
Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Val Ser Lys Asp Glu 675 680 685	
AAT CTA CTT AAA GCA TTT AAA GAA GAT TTA GAT ATT CAT ACA ATT ACT	2112
Asn Leu Leu Lys Ala Phe Lys Glu Asp Leu Asp Ile His Thr Ile Thr 690 695 700	
GCT GCC AAA ATT TTT GGT GTT TCA GAG ATG TTT GTT AGT GAA CAA ATG	2160
Ala Ala Lys Ile Phe Gly Val Ser Glu Met Phe Val Ser Glu Gln Met 705 710 715 720	

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AGA AGA GTT GGA AAG ATG GTA AAT TTT GCA ATT ATT TAT GGA GTT TCA	2208
Arg Arg Val Gly Lys Met Val Asn Phe Ala Ile Ile Tyr Gly Val Ser	
725 730 735	
CCT TAT GGT CTT TCA AAG AGA ATT GGT CTT AGT GTT TCA GAG ACT AAA	2256
Pro Tyr Gly Leu Ser Lys Arg Ile Gly Leu Ser Val Ser Glu Thr Lys	
740 745 750	
AAA ATA ATA GAT AAC TAT TTT AGA TAC TAT AAA GGA GTT TTT GAA TAT	2304
Lys Ile Ile Asp Asn Tyr Phe Arg Tyr Tyr Lys Gly Val Phe Glu Tyr	
755 760 765	
TTA AAA AGG ATG AAA GAT GAA GCA AGG AAA AAA GGT TAT GTT ACA ACG	2352
Leu Lys Arg Met Lys Asp Glu Ala Arg Lys Lys Gly Tyr Val Thr Thr	
770 775 780	
CTT TTT GGA AGG CGC AGA TAT ATT CCA CAG TTA AGA TCG AAA AAT GGT	2400
Leu Phe Gly Arg Arg Arg Tyr Ile Pro Gln Leu Arg Ser Lys Asn Gly	
785 790 795 800	
AAT AGA GTT CAA GAA GGA GAA AGA ATA GCT GTA AAC ACT CCA ATT CAA	2448
Asn Arg Val Gln Glu Gly Glu Arg Ile Ala Val Asn Thr Pro Ile Gln	
805 810 815	
GGA ACA GCA GCT GAT ATA ATA AAG ATA GCT ATG ATT AAT ATT CAT AAT	2496
Gly Thr Ala Ala Asp Ile Ile Lys Ile Ala Met Ile Asn Ile His Asn	
820 825 830	
AGA TTG AAG AAG GAA AAT CTA CGT TCA AAA ATG ATA TTG CAG GTT CAT	2544
Arg Leu Lys Lys Glu Asn Leu Arg Ser Lys Met Ile Leu Gln Val His	
835 840 845	
GAC GAG TTA GTT TTT GAA GTG CCC GAT AAT GAA CTG GAG ATT GTA AAA	2592
Asp Glu Leu Val Phe Glu Val Pro Asp Asn Glu Leu Glu Ile Val Lys	
850 855 860	
GAT TTA GTA AGA GAT GAG ATG GAA AAT GCA GTT AAG CTA GAC GTT CCT	2640
Asp Leu Val Arg Asp Glu Met Glu Asn Ala Val Lys Leu Asp Val Pro	
865 870 875 880	
TTA AAA GTA GAT GTT TAT TAT GGA AAA GAG TGG GAA TAA	2679
Leu Lys Val Asp Val Tyr Tyr Gly Lys Glu Trp Glu	
885 890	

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 892 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met	Gly	Lys	Met	Phe	Leu	Phe	Asp	Gly	Thr	Gly	Leu	Val	Tyr	Arg	Ala	1	5	10	15
Phe	Tyr	Ala	Ile	Asp	Gln	Ser	Leu	Gln	Thr	Ser	Ser	Gly	Leu	His	Thr	20	25	30	
Asn	Ala	Val	Tyr	Gly	Leu	Thr	Lys	Met	Leu	Ile	Lys	Phe	Leu	Lys	Glu	35	40	45	
His	Ile	Ser	Ile	Gly	Lys	Asp	Ala	Cys	Val	Phe	Val	Leu	Asp	Ser	Lys	50	55	60	
Gly	Gly	Ser	Lys	Lys	Arg	Lys	Asp	Ile	Leu	Glu	Thr	Tyr	Lys	Ala	Asn	65	70	75	80
Arg	Pro	Ser	Thr	Pro	Asp	Leu	Leu	Leu	Glu	Gln	Ile	Pro	Tyr	Val	Glu	85	90	95	
Glu	Leu	Val	Asp	Ala	Leu	Gly	Ile	Lys	Val	Leu	Lys	Ile	Glu	Gly	Phe	100	105	110	
Glu	Ala	Asp	Asp	Ile	Ile	Ala	Thr	Leu	Ser	Lys	Lys	Phe	Glu	Ser	Asp	115	120	125	
Phe	Glu	Lys	Val	Asn	Ile	Ile	Thr	Gly	Asp	Lys	Asp	Leu	Leu	Gln	Leu	130	135	140	
Val	Ser	Asp	Lys	Val	Phe	Val	Trp	Arg	Val	Glu	Arg	Gly	Ile	Thr	Asp	145	150	155	160
Leu	Val	Leu	Tyr	Asp	Arg	Asn	Lys	Val	Ile	Glu	Lys	Tyr	Gly	Ile	Tyr	165	170	175	
Pro	Glu	Gln	Phe	Lys	Asp	Tyr	Leu	Ser	Leu	Val	Gly	Asp	Gln	Ile	Asp	180	185	190	
Asn	Ile	Pro	Gly	Val	Lys	Gly	Ile	Gly	Lys	Lys	Thr	Ala	Val	Ser	Leu	195	200	205	
Leu	Lys	Lys	Tyr	Asn	Ser	Leu	Glu	Asn	Val	Leu	Lys	Asn	Ile	Asn	Leu	210	215	220	
Leu	Thr	Glu	Lys	Leu	Arg	Arg	Leu	Leu	Glu	Asp	Ser	Lys	Glu	Asp	Leu	225	230	235	240

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Gln Lys Ser Ile Glu Leu Val Glu Leu Ile Tyr Asp Val Pro Met Asp
 245 250 255
 Val Glu Lys Asp Glu Ile Ile Tyr Arg Gly Tyr Asn Pro Asp Lys Leu
 260 265 270
 Leu Lys Val Leu Lys Lys Tyr Glu Phe Ser Ser Ile Ile Lys Glu Leu
 275 280 285
 Asn Leu Gln Glu Lys Leu Glu Lys Glu Tyr Ile Leu Val Asp Asn Glu
 290 295 300
 Asp Lys Leu Lys Lys Leu Ala Glu Glu Ile Glu Lys Tyr Lys Thr Phe
 305 310 315 320
 Ser Ile Asp Thr Glu Thr Thr Ser Leu Asp Pro Phe Glu Ala Lys Leu
 325 330 335
 Val Gly Ile Ser Ile Ser Thr Met Glu Gly Lys Ala Tyr Tyr Ile Pro
 340 345 350
 Val Ser His Phe Gly Ala Lys Asn Ile Ser Lys Ser Leu Ile Asp Lys
 355 360 365
 Phe Leu Lys Gln Ile Leu Gln Glu Lys Asp Tyr Asn Ile Val Gly Gln
 370 375 380
 Asn Leu Lys Phe Asp Tyr Glu Ile Phe Lys Ser Met Gly Phe Ser Pro
 385 390 395 400
 Asn Val Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Asn Pro
 405 410 415
 Asp Glu Lys Arg Phe Asn Leu Glu Glu Leu Ser Leu Lys Tyr Leu Gly
 420 425 430
 Tyr Lys Met Ile Ser Phe Asp Glu Leu Val Asn Glu Asn Val Pro Leu
 435 440 445
 Phe Gly Asn Asp Phe Ser Tyr Val Pro Leu Glu Arg Ala Val Glu Tyr
 450 455 460
 Ser Cys Glu Asp Ala Asp Val Thr Tyr Arg Ile Phe Arg Lys Leu Gly
 465 470 475 480
 Arg Lys Ile Tyr Glu Asn Glu Met Glu Lys Leu Phe Tyr Glu Ile Glu
 485 490 495
 Met Pro Leu Ile Asp Val Leu Ser Glu Met Glu Leu Asn Gly Val Tyr
 500 505 510
 Phe Asp Glu Glu Tyr Leu Lys Glu Leu Ser Lys Lys Tyr Gln Glu Lys
 515 520 525
 Met Asp Gly Ile Lys Glu Lys Val Phe Glu Ile Ala Gly Glu Thr Phe
 530 535 540

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Asn 545	Leu	Asn	Ser	Ser	Thr	Gln	Val	Ala	Tyr	Ile 555	Leu	Phe	Glu	Lys	Leu 560
Asn	Ile	Ala	Pro	Tyr 565	Lys	Lys	Thr	Ala	Thr 570	Gly	Lys	Phe	Ser	Thr	Asn 575
Ala	Glu	Val	Leu 580	Glu	Glu	Leu	Ser	Lys 585	Glu	His	Glu	Ile	Ala 590	Lys	Leu
Leu	Leu	Glu 595	Tyr	Arg	Lys	Tyr	Gln 600	Lys	Leu	Lys	Ser	Thr 605	Tyr	Ile	Asp
Ser 610	Ile	Pro	Leu	Ser	Ile	Asn 615	Arg	Lys	Thr	Asn 620	Arg	Val	His	Thr	Thr
Phe 625	His	Gln	Thr	Gly	Thr 630	Ser	Thr	Gly	Arg	Leu 635	Ser	Ser	Ser	Asn	Pro 640
Asn	Leu	Gln	Asn 645	Leu	Pro	Thr	Arg	Ser	Glu 650	Glu	Gly	Lys	Glu	Ile	Arg 655
Lys	Ala	Val	Arg 660	Pro	Gln	Arg	Gln	Asp 665	Trp	Trp	Ile	Leu	Gly 670	Ala	Asp
Tyr	Ser	Gln 675	Ile	Glu	Leu	Arg	Val 680	Leu	Ala	His	Val	Ser 685	Lys	Asp	Glu
Asn 690	Leu	Leu	Lys	Ala	Phe	Lys 695	Glu	Asp	Leu	Asp	Ile 700	His	Thr	Ile	Thr
Ala 705	Ala	Lys	Ile	Phe	Gly 710	Val	Ser	Glu	Met	Phe 715	Val	Ser	Glu	Gln	Met 720
Arg	Arg	Val	Gly 725	Lys	Met	Val	Asn	Phe	Ala 730	Ile	Ile	Tyr	Gly	Val	Ser 735
Pro	Tyr	Gly 740	Leu	Ser	Lys	Arg	Ile	Gly 745	Leu	Ser	Val	Ser 750	Glu	Thr	Lys
Lys	Ile	Ile 755	Asp	Asn	Tyr	Phe	Arg 760	Tyr	Tyr	Lys	Gly	Val 765	Phe	Glu	Tyr
Leu 770	Lys	Arg	Met	Lys	Asp	Glu 775	Ala	Arg	Lys	Lys	Gly 780	Tyr	Val	Thr	Thr
Leu 785	Phe	Gly	Arg	Arg	Arg 790	Tyr	Ile	Pro	Gln	Leu 795	Arg	Ser	Lys	Asn	Gly 800
Asn	Arg	Val	Gln 805	Glu	Gly	Glu	Arg	Ile	Ala 810	Val	Asn	Thr	Pro	Ile 815	Gln
Gly	Thr	Ala	Ala 820	Asp	Ile	Ile	Lys	Ile 825	Ala	Met	Ile	Asn	Ile	His	Asn 830
Arg	Leu	Lys 835	Lys	Glu	Asn	Leu	Arg 840	Ser	Lys	Met	Ile	Leu	Gln	Val	His 845

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Asp Glu Leu Val Phe Glu Val Pro Asp Asn Glu Leu Glu Ile Val Lys
 850 855 860

Asp Leu Val Arg Asp Glu Met Glu Asn Ala Val Lys Leu Asp Val Pro
 865 870 875 880

Leu Lys Val Asp Val Tyr Tyr Gly Lys Glu Trp Glu
 885 890

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA probe BW33

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCGCTGCG CGTAACCACC ACACCCGCCG CGC

33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer BW37

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCGCTAGGGC GCTGGCAAGT GTAGCGGTCA

30

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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..4
- (D) OTHER INFORMATION: /label= Xaa
/note= "Xaa = Val or Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Xaa Tyr Gly
1

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Glu Ala Tyr Gly
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids

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(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Glu Ala Tyr Glu
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

(A) NAME/KEY: Peptide
(B) LOCATION: 1..4
(D) OTHER INFORMATION: /label= Xaa
/note= "Xaa = Leu or Ile"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Xaa Leu Glu Thr
1

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: internal
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION: 1..7
 - (D) OTHER INFORMATION: /label= Xaa
/note= "Xaa = Leu or Ile"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Xaa	Leu	Glu	Thr	Tyr	Lys	Ala
1				5		

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: internal
- (ix) FEATURE:
 - (A) NAME/KEY: Peptide
 - (B) LOCATION: 1..7
 - (D) OTHER INFORMATION: /label= Xaa1-4
/note= "Xaa1 = Ile or Leu or Ala; Xaa2-4, each =
any amino acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Xaa	Xaa	Xaa	Xaa	Tyr	Lys	Ala
1				5		

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(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer MK61

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGGACTACAA CTGCCACACA CC

22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer RA01

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGAGGCGCGC CAGCCCCAGG AGATCTACCA GTCCTTG

38

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer DG29

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCTTATGTC TCCAAAAGCT

20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer DG30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCTTTTGGA GACATA

16

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer PL10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCGTACCTT TGTCTCACGG GCAAC

25

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

-163-

(ii) MOLECULE TYPE: DNA primer FL63

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATAAAGGCA TGCTTCAGCT TGTGAACG

28

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer FL69

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGTACTTCTC TAGAAGCTGA ACAGCAG

27

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer FL64

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTGAAGCATG TCTTTGTCAC CGGTTACTAT CAATAT

36

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(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer FL65

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TAGTAACCGG TGACAAAG

18

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer FL66

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CTATGCCATG GATAGATCGC TTTCTACTTC C

31

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer FL67

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAAGCCCATG GAAACTTACA AGGCTCAAAG A

31

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer TZA292

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTCGGCATAT GGCTCCTGCT CCTCTTGAGG AGGCCCCCTG GCCCCCGCC

49

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer TZR01

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GACGCAGATC TCAGCCCTTG GCGGAAAGCC AGTCCTC

37

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA primer TSA288

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTCGGCATAT GGCTCCTAAA GAAGCTGAGG AGGCCCCCTG GCCCCCGCC

49

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer TSR01

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAGGCAGATC TCAGGCCTTG GCGGAAAGCC AGTCCTC

37

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer DG122

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTCTAAAGC GCAGATCTGA TATCAACCCT TGGCGGAAAG C

41

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(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer TAFI285

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTCGGCATAT GATTAAAGAA CTTAATTTAC AAGAAAAATT AGAAAAGG

48

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer TAFR01

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCTTTACCCC AGGATCCTCA TTCCCACTCT TTTCCATAAT AAACAT

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WHAT IS CLAIMED IS:

1. A recombinant thermostable DNA polymerase enzyme
which exhibits altered 5' to 3' exonuclease
5 activity from that of its native DNA polymerase.
2. The recombinant thermostable DNA polymerase enzyme
of claim 1 wherein a greater amount of 5' to 3'
exonuclease activity is exhibited than that of the
10 native DNA polymerase.
3. The recombinant thermostable DNA polymerase enzyme
of claim 2 comprising the amino acid sequence
A(X)YG wherein X is V or T (SEQ ID NO:15), and/or
15 the amino acid sequence $X_A X_3 YKA$ wherein X_A is I, L
or A and X_3 is any sequence of three amino acids
(SEQ ID NO:20).
4. The recombinant thermostable DNA polymerase enzyme
20 of claim 1 wherein a lesser amount of 5' to 3'
exonuclease activity is exhibited than that of the
native DNA polymerase.
5. The recombinant thermostable DNA polymerase enzyme
25 of claim 4 which in its native form comprises the
amino acid sequence A(X)YG wherein X is V or T (SEQ
ID NO:15), said amino acid sequence being mutated
or deleted in said recombinant enzyme.
- 30 6. The recombinant thermostable DNA polymerase enzyme
of claim 5 wherein G of SEQ ID NO:15 is mutated.
7. The recombinant thermostable DNA polymerase enzyme
of claim 6 wherein G of SEQ ID NO:15 is mutated to
35 A.

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8. The recombinant thermostable DNA polymerase enzyme of claim 4 which in its native form comprises the amino acid sequence HEAYG (SEQ ID NO:16), said amino acid sequence being mutated or deleted in
5 said recombinant enzyme.
9. The recombinant thermostable DNA polymerase enzyme of claim 4 which in its native form comprises the amino acid sequence HEAYE (SEQ ID NO:17), said
10 amino acid sequence being mutated or deleted in said recombinant enzyme.
10. The recombinant thermostable DNA polymerase enzyme of claim 4 which in its native form comprises the
15 amino acid sequence XLET wherein X is L or I (SEQ ID NO:18), said amino acid sequence being mutated or deleted in said recombinant enzyme.
11. The recombinant thermostable DNA polymerase enzyme
20 of claim 4 selected from the group consisting of mutant forms of Thermus species sps17, Thermus species Z05, Thermus aquaticus, Thermus thermophilus, Thermosipho africanus and Thermotoga maritima.
- 25 12. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus comprising amino acids 77-832 of SEQ ID NO:2.
- 30 13. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus comprising amino acids 47-832 of SEQ ID NO:2.

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14. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus comprising amino acids 155-832 of SEQ ID NO:2.
- 5
15. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus comprising amino acids 203-832 of SEQ ID NO:2.
- 10
16. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus comprising amino acids 290-832 of SEQ ID NO:2.
- 15
17. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima comprising amino acids 38-893 of SEQ ID NO:4.
- 20
18. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima comprising amino acids 21-893 of SEQ ID NO:4.
- 25
19. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima comprising amino acids 74-893 of SEQ ID NO:4.
- 30
20. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima comprising amino acids 140-893 of SEQ ID NO:4.
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21. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima comprising amino acids 284-893 of SEQ ID NO:4.

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22. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17 comprising amino acids 44-830 of SEQ ID NO:6.

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23. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17 comprising amino acids 74-830 of SEQ ID NO:6.

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24. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17 comprising amino acids 152-830 of SEQ ID NO:6.

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25. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17 comprising amino acids 200-830 of SEQ ID NO:6.

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26. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17 comprising amino acids 288-830 of SEQ ID NO:6.

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27. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05 comprising amino acids 47-834 of SEQ ID NO:8.

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28. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05 comprising amino acids 78-834 of SEQ ID NO:8.
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29. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05 comprising amino acids 156-834 of SEQ ID NO:8.
- 10
30. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05 comprising amino acids 204-834 of SEQ ID NO:8.
- 15
31. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05 comprising amino acids 292-834 of SEQ ID NO:8.
- 20
32. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus comprising amino acids 47-834 of SEQ ID NO:10.
- 25
33. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus comprising amino acids 78-834 of SEQ ID NO:10.
- 30
34. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus comprising amino acids 156-834 of SEQ ID NO:10.
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35. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus comprising amino acids 204-834 of SEQ ID NO:10.

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36. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus comprising amino acids 292-834 of SEQ ID NO:10.

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37. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus comprising amino acids 38-892 of SEQ ID NO:12.

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38. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus comprising amino acids 94-892 of SEQ ID NO:12.

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39. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus comprising amino acids 140-892 of SEQ ID NO:12.

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40. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus comprising amino acids 204-892 of SEQ ID NO:12.

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41. The recombinant thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus comprising amino acids 285-892 of SEQ ID NO:12.

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42. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus, said DNA sequence comprising nucleotides 229-2499 of SEQ ID NO:1.
43. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus, said DNA sequence comprising nucleotides 139-2499 of SEQ ID NO:1.
44. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus, said DNA sequence comprising nucleotides 463-2499 of SEQ ID NO:1.
45. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus, said DNA sequence comprising nucleotides 607-2499 of SEQ ID NO:1.
46. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus aquaticus, said DNA sequence comprising nucleotides 868-2499 of SEQ ID NO:1.
47. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima, said DNA sequence comprising nucleotides 132-2682 of SEQ ID NO:3.

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48. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima, said DNA sequence comprising nucleotides 61-2682 of SEQ ID NO:3.
49. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima, said DNA sequence comprising nucleotides 220-2682 of SEQ ID NO:3.
50. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima, said DNA sequence comprising nucleotides 418-2682 of SEQ ID NO:3.
51. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermotoga maritima, said DNA sequence comprising nucleotides 850-2682 of SEQ ID NO:3.
52. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17, said DNA sequence comprising nucleotides 130-2493 of SEQ ID NO:5.
53. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17, said DNA sequence comprising nucleotides 220-2493 of SEQ ID NO:5.

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54. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17, said DNA sequence comprising nucleotides 454-2493 of SEQ ID NO:5.
55. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17, said DNA sequence comprising nucleotides 598-2493 of SEQ ID NO:5.
56. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species sps17, said DNA sequence comprising nucleotides 862-2493 of SEQ ID NO:5.
57. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05, said DNA sequence comprising nucleotides 139-2505 of SEQ ID NO:7.
58. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05, said DNA sequence comprising nucleotides 232-2505 of SEQ ID NO:7.
59. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05, said DNA sequence comprising nucleotides 476-2505 of SEQ ID NO:7.

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60. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05, said DNA sequence comprising nucleotides 610-2505 of SEQ ID NO:7.
61. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus species Z05, said DNA sequence comprising nucleotides 874-2505 of SEQ ID NO:7.
62. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus, said DNA sequence comprising nucleotides 139-2505 of SEQ ID NO:9.
63. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus, said DNA sequence comprising nucleotides 232-2505 of SEQ ID NO:9.
64. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus, said DNA sequence comprising nucleotides 466-2505 of SEQ ID NO:9.
65. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus, said DNA sequence comprising nucleotides 610-2505 of SEQ ID NO:9.

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66. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermus thermophilus, said DNA sequence comprising nucleotides 874-2505 of SEQ ID NO:9.
67. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus, said DNA sequence comprising nucleotides 112-2679 of SEQ ID NO:11.
68. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus, said DNA sequence comprising nucleotides 280-2679 of SEQ ID NO:11.
69. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus, said DNA sequence comprising nucleotides 418-2679 of SEQ ID NO:11.
70. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus, said DNA sequence comprising nucleotides 610-2679 of SEQ ID NO:11.
71. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 11 wherein said enzyme is a mutant form of Thermosipho africanus, said DNA sequence comprising nucleotides 853-2679 of SEQ ID NO:11.

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72. A DNA sequence which encodes a thermostable DNA polymerase enzyme of claim 3.
73. A DNA sequence which encodes a thermostable DNA polymerase enzyme of any of claim 5 through 10.
74. A recombinant DNA vector comprising the DNA sequence of any of claims 42 through 73.
- 10 75. A recombinant host cell transformed with the vector of claim 74.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/07035

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1.5	C 12 N 15/54	C 12 N 9/12	C 12 N 1/21
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II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1.5	C 12 N

**Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category °	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	The FASEB Journal, volume 5, no. 4, March 1991, (Bethesda, MD, US) R.D. Abramson et al.: "Characterization of the 5'-3' exonuclease activity of thermus aquaticus DNA polymerase", page A437, abstract no. 386, see the abstract ---	1,4
P,Y	---	11-16
P,X	WO,A,9109944 (CETUS CORP.) 11 July 1991, see page 13, lines 28-34; page 14, lines 7-25; page 14, line 34 - page 16, line 15: claims 1,2,5 (cited in the application) --- -/-	1,4

° Special categories of cited documents : ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed


"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 29-11-1991	Date of Mailing of this International Search Report 16 JAN 1992
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Mme N. KUIPER 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	---	1-11,17 -21,32- 36,42- 51,62- 66,72- 75
P,Y	WO,A,9109950 (CETUS CORP.) 11 July 1991, see claims 3,4 (cited in the application)	1-7,9, 11,32- 36,62- 66,72- 75
P,X	---	
P,Y	WO,A,9102090 (PROMEGA CORP.) 21 February 1991, see claim 1; page 6, line 34 - page 7, line 7	1,4,5, 11,14- 16,73 6-10,12 ,13,17- 72,74, 75
Y	---	
Y	The Journal of Biological Chemistry, volume 264, no. 11, 15 April 1989, Am. Soc. for Biochemist. and Molecular Biology, Inc. (US) F.C: Lawyer et al.: "Isolation, characterization, and expression in Eschericia coli of the DNA polymerase gene from thermus aquaticus", pages 6427-6437, see figure 2; page 6432, lines 25-30 (cited in the application)	1-8,11- 16,42- 46,72- 75
Y	---	
Y	Cell, volume 59, no. 1, 6 October 1989, Cell Press (MA, US) A. Bernad et al.: "A conserved 3'-5' exonuclease active site in prokaryotic and eukaryotic DNA polymerases", pages 219-228, see page 224, lines 6-10, line 21 - page 225, line 14; figure 5	1,4-75
Y	---	
Y	Proc. Natl. Acad. Sci., volume 86, no. 12, June 1989, Biochemistry (US) M.C. Leavitt et al.: "T5 DNA polymerase: structural-functional relationships to other DNA polymerases", pages 4465-4469, see figure 4; page 4468, lines 13-35	1,4-75

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Chemical Abstracts, volume 93, no. 5, 4 August 1980 (Columbus, Ohio, US) A.S. Kaledin et al.: "Isolation and properties of DNA polymerase from extremal thermophilic bacteria <i>Thermus aquaticus</i> YT-1", see page 377, abstract 40169p, & Biokhimiya (Moscow) 1980, 45(4), 644-51 ---	1,4
A	Chemical Abstracts, volume 85, no. 21, 22 November 1976, (Columbus, Ohio, US) A. Chien et al.: "Deoxyribonucleic acid polymerase from the extreme thermophile <i>Thermus aquaticus</i> ", see page 180, abstract 155559t, & J. Bacteriol. 1976, 127(3), 550-7 -----	1,4

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9107035
SA 52103

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 13/12/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9109944	11-07-91	WO-A- 9109950	11-07-91
WO-A- 9109950	11-07-91	WO-A- 9109944	11-07-91
WO-A- 9102090	21-02-91	AU-A- 6341190	11-03-91